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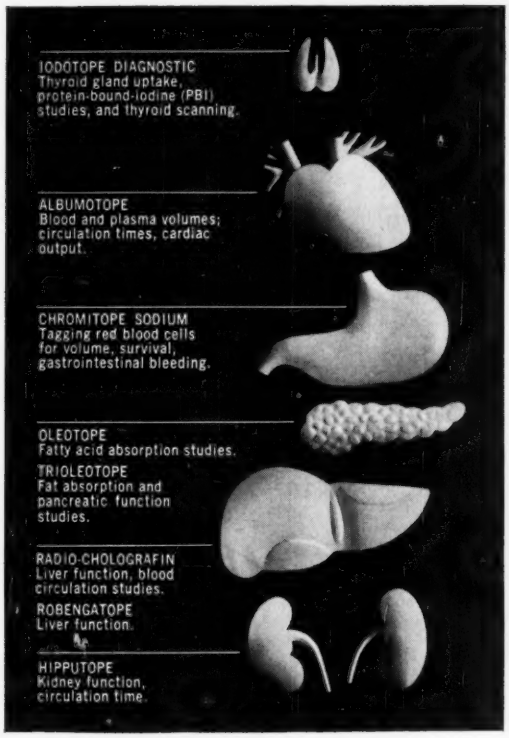

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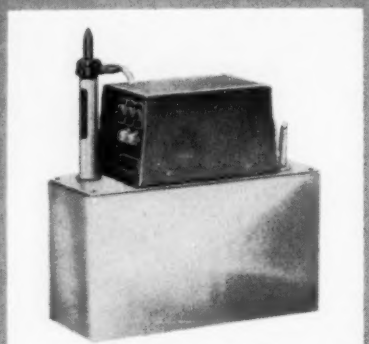

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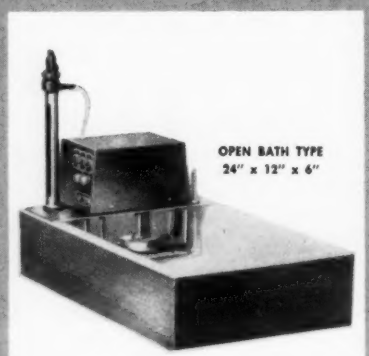

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[1]Lathe, G. H. (Sass-Kortsak, Andrew, ed.) in **Kernicterus;** report based on symposium held at IX International Congress of Paediatrics, Montreal, July, 1959. University of Toronto Press, Toronto, 1961.

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Why arthritic patients feel much better on Dianabol

1. In arthritis, Dianabol improves general physical condition

Arthritis, like other chronic illnesses, plunges the body into a catabolic state. Protein stores are depleted; appetite wanes; weight drops; strength and vigor decline. By aiding the deposition, synthesis and utilization of protein, and by conserving calcium, Dianabol promotes lean weight gain, increases strength and vitality, and strengthens bone structure in patients with a wide range of chronic diseases. Recent studies show that adjunctive use of Dianabol may be particularly valuable in patients with arthritis to improve over-all clinical status. Kuzell and Naugler,[1] for example, report that arthritic patients on Dianabol "generally have experienced an increase in appetite, weight, strength and endurance." Kuzell and Naugler note further: "Unlike some other testosterone derivatives, the use of this compound [Dianabol] is not followed by virilizing phenomena. Fluid retention has been no problem."

2. In arthritis, Dianabol helps restore a sense of well-being

Plagued by pain and reduced mobility, arthritics often lose hope and become depressed. The marked improvement in general health usually associated with therapy with Dianabol may have a favorable effect in these patients, as it has in so many chronically ill individuals. As physical status improves, hope is revived and a sense of well-being restored. Commenting on the use of Dianabol in a group of debilitated, cachectic patients, Gingrich[2] states: "The majority of patients experienced increase in appetite and a feeling of well-being."

3. In arthritis, Dianabol augments the beneficial effects of salicylates, corticosteroids, etc.

Several investigators[1,3,4] have observed improved therapeutic response after the addition of Dianabol to antiarthritis regimens. Kuzell and Naugler[1] state: "In generalized osteoarthritis, symptoms have been less bothersome, and in ankylosing spondylitis gain in weight and strength has followed the use of Dianabol." Clark,[3] reporting on 12 hospitalized patients with rheumatoid arthritis being given moderate to large doses of corticosteroids with evidence of steroid intoxication, noted that the addition of Dianabol promptly decreased joint symptoms but increased steroid intoxication. However, with Dianabol it was possible to reduce corticosteroid dosage considerably, while maintaining and even furthering clinical improvement. In 15 ambulatory patients on small maintenance doses of corticosteroids, the addition of Dianabol resulted in further clinical improvement which was continued even when corticosteroid dosage was reduced in some cases.[3,4]

4. In arthritis, Dianabol counteracts the catabolic effects of corticosteroids

Prolonged use of corticosteroids may result in excessive breakdown of protein in all tissues, including bone,[5] as well as undue phosphorus and calcium loss.[6] If protein destruction is allowed to go unchecked, it may lead to osteoporosis—a condition that has occurred with increasing frequency in patients receiving corticosteroids for extended periods.[7]
Tillis[5] asserts that it is "imperative" to restore the protein bone matrix in such patients

through the use of an anabolic agent. He studied the specific anabolic benefits of Dianabol in 50 patients with osteoporosis (34 postmenopausal and 16 corticosteroid-induced), most of whom also had rheumatoid arthritis. Dianabol relieved bone pain, increased strength and vigor, and induced a sense of well-being in 41 (82 per cent) of these patients. Edema, observed in 8 patients, was cleared in 4 by reduction of dosage; the remaining 4 responded promptly to hydrochlorothiazide. Gastric distress was noted in 2 patients, slight hoarseness in 1 woman, and facial acne in 1 woman. Other investigators[8,9] have shown that addition of Dianabol to the regimens of patients receiving corticosteroids improved nitrogen and potassium metabolism and reduced phosphorus and calcium losses. Reporting on 10 patients taking corticosteroids, most of whom had corticosteroid-induced osteoporosis and/or myopathy, Abbott[9] states: "In the patients who showed a markedly negative nitrogen balance the administration of 10 mg. of Dianabol per day greatly reduced the protein deficit. In others who were eating well and taking smaller amounts of corticosteroids a positive nitrogen balance resulted which increased with Dianabol." Abbott notes that creatinuria, which occurred on corticosteroids alone, was increased by Dianabol, as it is by methyltestosterone and the newer oral methyl- or ethyl-testosterone derivatives. However, he observes that the "significance of this creatinuria is not known and no ill effects have been ascribed to this change." While the finding of elevated serum aldolase levels raised the theoretical possibility of potentially deleterious effects, Vignos *et al*[8] and Abbott[9] noted no androgenic or myopathic effects and no liver disorders in patients who took Dianabol and corticosteroids for up to 8 months. Kuzell and Naugler[1] state it is their impression that Dianabol has checked weight loss following prolonged administration of triamcinolone in patients with rheumatoid arthritis. They add that, with Dianabol, protein patterns have migrated toward normal profiles, purpura consequent to corticosteroid administration has been lessened, and the erythrocyte sedimentation rate has been diminished.

REFERENCES: 1. Kuzell, W. C., and Naugler, W. E.: Paper presented at the Annual Meeting of the American Rheumatism Association, Hollywood-By-The-Sea, Florida, June 9-11, 1960. 2. Gingrich, G. W.: Clinical report to CIBA. 3. Clark, G. M.: Paper presented at the Seventh Interim Session of the American Rheumatism Association, Dallas, Texas, Dec. 10, 1960. 4. Clark, G. M., Kaplan, S., Goobar, J., and Mills, D.: Arthritis and Rheumatism *4*:106 (Feb.) 1961. 5. Tillis, H. H.: Clin. Med. *8*:274 (Feb.) 1961. 6. Lockie, L. M.: J.A.M.A. *170*:1063 (June 27) 1959. 7. Boland, E. W.: J.A.M.A. *150*:1281 (Nov. 29) 1952. 8. Vignos, P. J., Jr., Abbott, W. E., Post, R. S., and Levy, S.: J. Lab. & Clin. Med. *56*:954 (Dec.) 1960. 9. Abbott, W. E.: Research report to CIBA. 10. Misurale, F.: Minerva med. *51*:996 (March 21) 1960.

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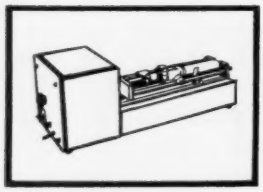

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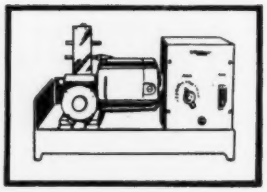

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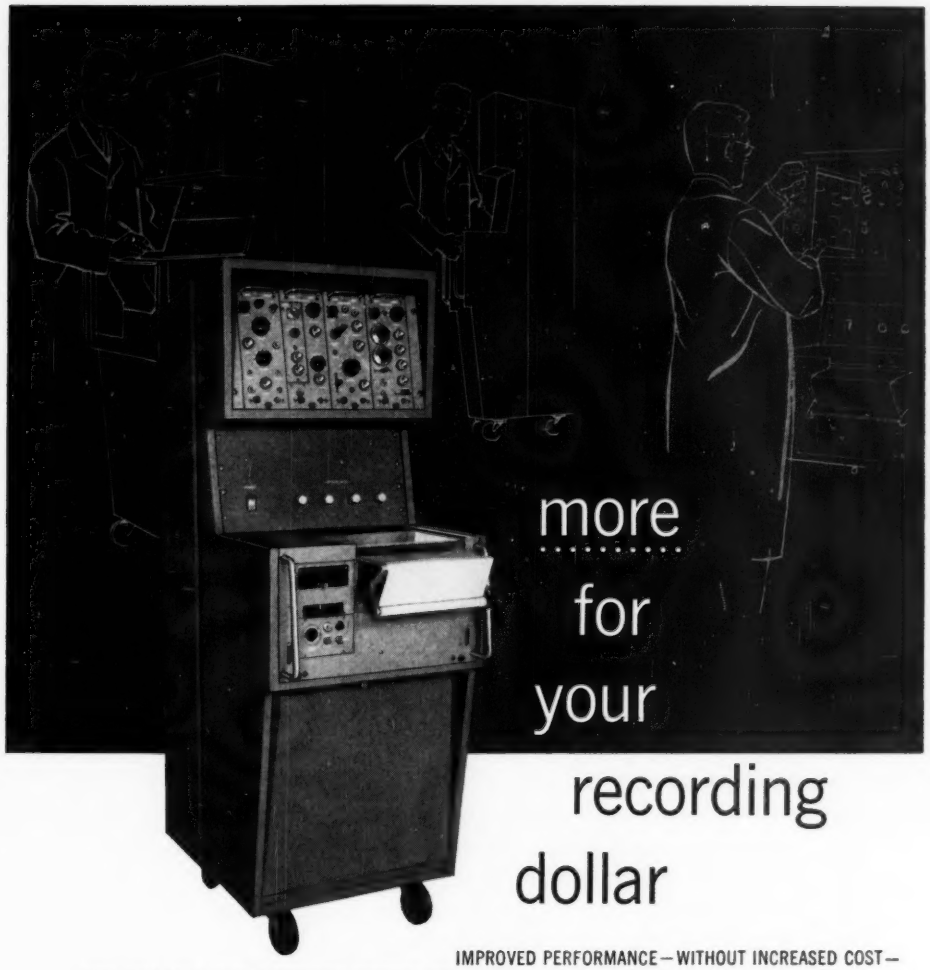

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(1) Matthias, J. Q.; Misiewicz, J. J., and Scott, R. B.: Brit. M. J. *2*:1837-1840 (Dec. 24) 1960.
(2) Coggins, P. R.; Ravdin, R. G., and Eisman, S. H.: Cancer *13*:1254-1260 (Nov.-Dec.) 1960.
(3) Papac, R.; Petrakis, N. L.; Amini, F., and Wood, D. A.: J.A.M.A. *172*:1387-1391 (March 26) 1960.

DOSAGE: *For neoplasms relatively susceptible to Cytoxan* — Patients with lymphomas and other neoplasms believed to be relatively susceptible to Cytoxan therapy are given an initial dose of 2-3 mg./Kg./day intravenously. White blood counts and platelet determinations should be made daily or twice weekly and the dosage adjusted accordingly. Intravenous infusions should be continued for at least 6 days unless otherwise indicated. A leukopenia of between 1500 and 5000 cells per cu. mm. (or lower) may be expected between the tenth and fourteenth day. In the presence of a leukopenia of less than 2000/cu. mm. Cytoxan should be discontinued until the white cell count returns to 2000 to 5000 (usually within a week). Dosage is subsequently adjusted as indicated by the patient's objective response and the leukocyte count. If the patient is subjectively improved, if the size of the tumor has decreased, or if the white cells are satisfactorily maintained between 2000 and 5000/cu. mm. oral dosage may be instituted equivalent to intravenous dosage.

Thrombocytopenia is rarely observed on this regimen. If platelet counts of less than 100,000/cu. mm. are observed, the patient should be watched carefully. If platelets continue to decrease, Cytoxan should be discontinued.

The patient who has had previous treatment with alkylating agents, or x-ray, or is debilitated may be more susceptible to bone marrow depression, and initial Cytoxan doses should be more conservative than the above. Such patients should have more frequent hematologic evaluation. Good medical practice demands access to a reliable hematologic laboratory when using Cytoxan.

For neoplasms relatively resistant to Cytoxan — Patients with carcinomas and other malignant neoplasms believed to be less susceptible to Cytoxan therapy are given a dose of 4 to 8 mg./Kg./day intravenously. Unless there are indications to the contrary, this dose is continued for 6 days, then stopped. Leukopenia usually ensues on the tenth to fourteenth day after the first dose of Cytoxan. Thrombocyte reduction is not common, and platelets may actually increase. The leukocyte count promptly returns toward normal levels in most cases, and as it begins to increase, sufficient Cytoxan is administered to maintain it near 2000 to 5000/cu. mm. This may be accomplished by two intravenous injections weekly, or by oral administration, or by a combination of both routes. An oral dosage of 50 to 200 mg. daily or an intravenous injection of 5 mg./Kg. twice weekly will usually suffice.

The platelet and leukocyte counts should be followed carefully, and the prior treatment history of patients carefully evaluated as delineated above.

Leukopenia as a guide to adequacy of dosage — The best objective measure for dosage seems to be the number of circulating white blood cells. This is used as an index of the activity of the hematopoietic system, especially the bone marrow. The mechanism by which Cytoxan causes a reduction in the level of white blood cells is not known, but cessation of dosage results in an increase in the level, indicating that the hematopoietic system had not been permanently affected. When large doses (8 mg./Kg./day for 6 days) are given initially, the white cell count falls rapidly. Following the cessation of the 6-day course, the white cells may continue to decline for as long as 8 days and then increase. The reduction of the white cell count during Cytoxan therapy and its subsequent increase when therapy is discontinued can be repeated in the same patient. Maximal reduction in leukocyte count indicates the maximal permissible Cytoxan level for therapeutic effect. Leukopenic patients must be watched carefully for evidence of infection.

Total white blood cell and thrombocyte counts should be obtained 2 or more times weekly in order to evaluate therapy and to adjust dosage.

SIDE EFFECTS: Although Cytoxan is related to nitrogen mustard, it has no vesicant effect on tissue. It does not traumatize the vein when injected intravenously, nor does it cause any localized tissue reaction following extravasation. It may be administered intravenously, intramuscularly, intraperitoneally, intrapleurally or directly into the tumor, when indicated. It is apparently active by each of these routes.

Nausea and vomiting are common and depend on dose and on individual susceptibility. However, many investigators accept the nausea and vomiting in favor of maintaining maximal therapy. The vomiting can be controlled with antiemetic agents.

Alopecia is a frequent side reaction to Cytoxan therapy. It has been observed in 28% of the patients studied in this country. The incidence is greater with larger doses. The loss of hair may first be noted about the 21st day of therapy and may proceed to alopecia totalis. This effect is reversed following discontinuance of Cytoxan; during reduced maintenance therapy, hair may reappear. It is essential to advise the patient in advance concerning this effect of the drug.

Dizziness of short duration and of minor degree has occasionally been reported.

Leukopenia is an expected effect and can be used as a guide to therapy. Thrombocytopenia may occur, especially after large doses. The leukocyte or platelet counts of an occasional patient may fall precipitously after even small doses of Cytoxan, as with all alkylating agents. The drug should be discontinued in such patients and reinstituted later at lower dosage after satisfactory hematologic recovery has occurred. Prior treatment with x-ray or with other chemotherapeutic agents frequently causes an earlier or exaggerated leukopenia or thrombocytopenia after Cytoxan medication. Only rarely has there been a report of erythrocyte or hemoglobin reduction.

ADMINISTRATION: Add 5 cc. sterile water (Water for Injection, U.S.P.) to 100 mg. of Cytoxan in the sterile vial (add 10 cc. to 200 mg. vial). Shake, allow to stand until clear, remove with sterile syringe and needle and inject.

The freshly prepared solution of Cytoxan may be administered intravenously, intramuscularly, intraperitoneally, intrapleurally, or directly into the tumor. The solution should be administered promptly after being made but is satisfactory for use for three hours after preparation.

If the patient is receiving a parenteral infusion, the Cytoxan solution may be injected into the rubber tubing if the solution is glucose or saline.

No thrombosis or thrombophlebitis has been reported from injections of Cytoxan. Extravasation of the drug into the subcutaneous tissues does not result in local reactions.

PRECAUTIONS: Cytoxan should not be given to any person with a severe leukopenia, thrombocytopenia, or bone marrow infiltrated with malignant cells. It may be given with suitable precautions to patients who have had recent x-ray treatment, recent treatment with a cytotoxic agent, a surgical procedure within 2-3 weeks, or debilitated patients.

AVAILABILITY: Cytoxan is available as follows:

Cytoxan for Injection, 100 mg., a sterile dry-filled vial containing 100 mg. cyclophosphamide and 45 mg. sodium chloride. Packaged, 12 vials per carton.

Cytoxan for Injection, 200 mg., a sterile dry-filled vial containing 200 mg. cyclophosphamide and 90 mg. sodium chloride. Packaged, 12 vials per carton.

Cytoxan Tablets for oral administration, 50 mg., white, round tablets, flecked with blue for easy identification. Packaged, 100 tablets per bottle.

For a copy of the Cytoxan brochure, or other additional information on Cytoxan, communicate directly with the Medical Department, Mead Johnson Laboratories, Evansville 21, Indiana.

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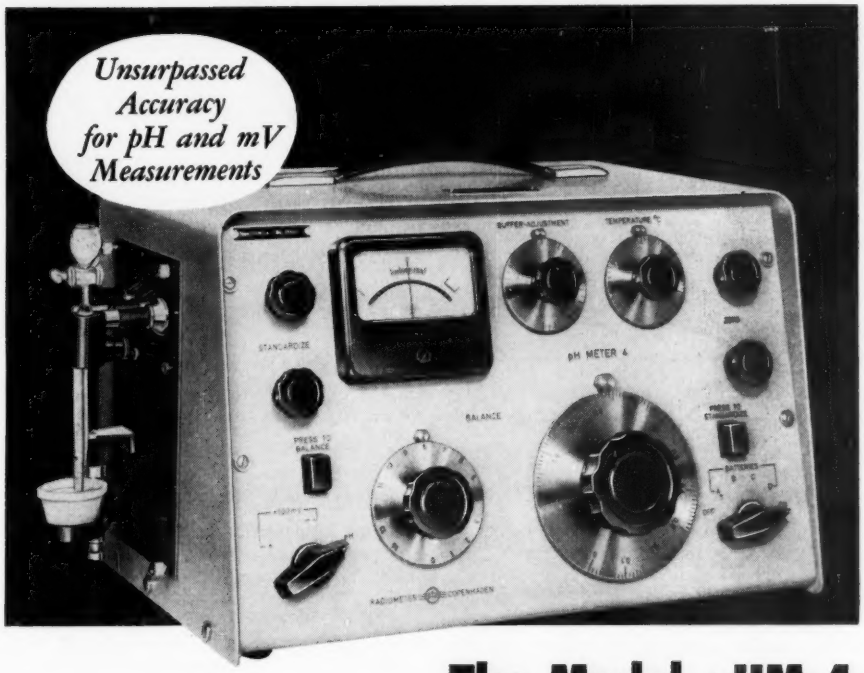

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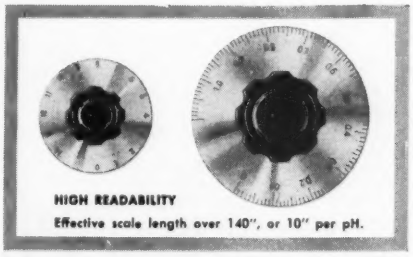

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ABSTRACTS

Babson, A. L., Read, P. A., and Phillips, G. E.: The importance of the substrate in assays of acid phosphatase in serum. Am. J. of Clin. Path. 32:1 83-87, July 1959.

A comparison is made between six generally used substrates for their relative specificity to prostatic and erythrocytic acid phosphatase. Results showed that a new substrate, alpha-naphthyl phosphate, was twice as specific as beta-glycerophosphate and 40-100 times as specific as all other substrates investigated. Acid phosphatase assays in serum should be specific for that enzyme arising from cancerous prostatic tissue. The substrate alpha-naphthyl phosphate* is specific for this enzyme.

Babson, A. L., and Read, P. A.: A new assay for prostatic acid phosphatase in serum. Am. J. Clin. Path. 32:1, 88-91, July 1959.

A new and specific method for the determination of prostatic acid phosphatase in serum is presented. The method is based on the use of a new substrate alpha-naphthyl phosphate which measures only prostatic acid phosphatase. The method is simple and rapid to perform with a minimum of manipulation. In this procedure the color developed represents prostatic enzyme activity alone. The reagents for this new method are available in convenient and stable form.*

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THE INFLUENCE OF BARBITURATES ON COUMARIN PLASMA LEVELS AND PROTHROMBIN RESPONSE *

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(Submitted for publication April 25, 1961; accepted May 19, 1961)

Wide individual variations in response to drugs is a common clinical problem, and is especially evident with coumarin anticoagulant therapy (1–3). A possible contributing factor to such variations could be the influence of another presumably unrelated drug upon the response to the coumarin anticoagulant. Reports from several laboratories (4–7) indicate that barbiturates can reduce the prothrombin response to coumarin anticoagulants. This report presents initial results of studies designed to determine the manner by which barbiturates alter the response to coumarins in man, guinea pig and dog.

MATERIAL AND METHODS

Guinea pigs. Diet, method of handling guinea pigs, and blood sample collection have been reported (8). Drugs were administered to guinea pigs intraperitoneally, barbital and acenocoumarin as aqueous solutions of their sodium salts in 0.5 to 1 ml, and chlorobutanol in 1.0 ml of peanut oil.

Dogs. Dogs were maintained on Kibbled dog food (Big Red Co.). They were given biscoumacetate (75 mg per kg) intravenously and barbital orally (50 mg per kg).

Human subjects. Ambulatory, hospitalized subjects free from known liver, kidney or gastrointestinal disease, on a regular hospital diet were studied. Their age ranged from 40 to 60 years. Heptabarbital and acenocoumarin were administered orally. Dicumarol (bishydroxycoumarin) and biscoumacetate were administered either orally or intravenously.

Chemical methods. The procedures for the estimation of prothrombin time (9), Dicumarol (10), and biscoumacetate (11) have been described. Acenocoumarin was determined by the method used for biscoumacetate except that the final extraction was made into a smaller volume (2 ml) of NaOH, and optical density was measured at 302 mμ. Barbital and heptabarbital did not interfere with the analyses of Dicumarol, biscoumacetate and acenocoumarin.

* Presented in part before the American Heart Association, Philadelphia, Pa., October 25, 1959. This investigation was supported in part by Research Grant H-4347 (C 1), National Heart Institute, United States Public Health Service, Bethesda, Md., and the National Vitamin Foundation, New York, N. Y.

RESULTS

Studies with guinea pigs. Administration of several daily doses of 140 mg per kg of sodium barbital to guinea pigs had no effect on prothrombin time (Table I). Administration of acenocoumarin produced the expected hypo-prothrombinemia observed previously (8). However, in animals which had received acenocoumarin after pretreatment with barbital, no appreciable hypoprothrombinemia was noted. At this dosage level of barbital the effect was consistent; with lower doses (100 mg per kg) block of acenocoumarin effect was less complete. Pretreatment with three daily doses of chlorobutanol (125 mg per kg) also antagonized the hypo-prothrombinemia induced by acenocoumarin. The average prothrombin time in the acenocoumarin-treated control group was 70 seconds (whole plasma) and 53 seconds in the chlorobutanol- and acenocoumarin-treated group. The corresponding time was 145 seconds and 84

TABLE I

Prothrombin response of guinea pigs to acenocoumarin, barbital, and acenocoumarin and barbital

	Plasma prothrombin time*			
	Whole		Saline diluted (12.5%)	
	Average	Range	Average	Range
	sec		*sec*	
Saline (1 ml)	38	32–45	54	44–67
Barbital†	38	32–43	62	55–70
Acenocoumarin‡	138	60–250	>200	97–420+
Acenocoumarin‡ and barbital†	41	33–61	80	55–128

* Blood collected on Day 5; nine animals in each group.
† Sodium barbital, 140 mg/kg, i.p. daily on Days 1–4.
‡ Sodium acenocoumarin, 4.5 mg, i.p. on Days 3 and 4 in 1 ml water.

TABLE II

Effect of pretreatment with barbital on plasma levels of acenocoumarin in guinea pigs *

Time	No pretreatment			Pretreated†		
	No. of animals	Plasma level		No. of animals	Plasma level	
		Mean	Range		Mean	Range
hrs		*mg/L*			*mg/L*	
0.5	5	40	25–56	8	26	18–42
1				7	16	7–24
1.5	11	34	16–51	9	6.6	2–12

* All animals were injected i.p. with 33 mg/kg of sodium acenocoumarin, and sacrificed at the indicated time.
† Injected with single daily i.p. doses of 140 mg/kg of sodium barbital beginning 4 days prior to the experiment.

seconds for diluted plasma. Chlorobutanol by itself had no effect on prothrombin time.

Barbital has a half-life in plasma of about 3 days in guinea pigs, and is not appreciably metabolized (12). The half-life of acenocoumarin, as measured by the fall in plasma concentration in guinea pigs, is much shorter than that of barbital. Considerably lower levels of acenocoumarin were found in barbital-pretreated animals than in control animals (Table II). In both groups of animals detectable amounts of acenocoumarin were absent in the plasma at the time of prothrombin estimation (24 hours after dosage).

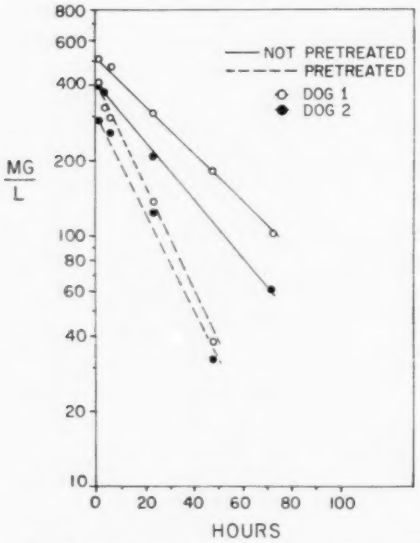

FIG. 1. PLASMA LEVELS IN TWO DOGS FOLLOWING AN INTRAVENOUS DOSE (75 MG/KG) OF BISCOUMACETATE. The same dose was repeated in the same animal several weeks later, 2 hours after an oral dose of barbital (50 mg/kg).

Studies in dogs. Plasma levels of biscoumacetate after a single intravenous dose of the drug were compared with levels resulting from the same dose after pretreatment of the same animals with barbital (Figure 1). Pretreatment accelerated the decline of the plasma biscoumacetate level and reduced the hypo-prothrombinemic response. Diluted (12.5 per cent) plasma prothrombin time 48 hours after dosage was 75 seconds (Dog 1) and 66 seconds (Dog 2) without pretreatment, and 65 and 48 seconds with pretreatment.

Studies in man. The prothrombin response was measured in the same human subjects after: *1*) an oral dose of a coumarin anticoagulant only;

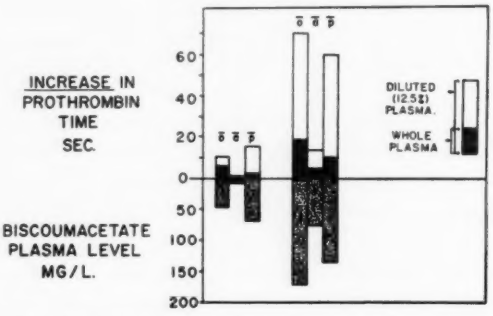

FIG. 2. MAXIMAL PROTHROMBIN RESPONSE (UPPER BARS) AND BISCOUMACETATE PLASMA LEVEL 5 HOURS AFTER A SINGLE ORAL DOSE OF THE DRUG (LOWER BARS) IN TWO ILLUSTRATIVE HUMAN SUBJECTS. The first set of bars presents values obtained in a relatively resistant subject (Wi), and the second set in a sensitive subject (M). The first bar in each set ($\bar{o}$), results without barbiturate; the center bar ($\bar{a}$), pretreated with 400 mg heptabarbital 1 hour before the anticoagulant; the last bar ($\bar{p}$), heptabarbital 5 hours after the anticoagulant.

2) pretreatment with an orally administered barbiturate (heptabarbital) followed by an orally administered coumarin; *3*) an orally administered coumarin, followed by an orally administered barbiturate; *4*) an intravenous dose of a coumarin only; *5*) pretreatment with an orally administered barbiturate followed by an intravenously administered coumarin. Experiments on individual subjects were carried out at 2- to 3-week intervals.

Results of experiments with biscoumacetate are shown in Figure 2 and Table III; with Dicumarol in Table IV and with acenocoumarin in Table V. Pretreatment with heptabarbital, followed by an *oral* dose of any of the three coumarin anticoagulants resulted in lower plasma levels of the cou-

TABLE III

Effect of heptabarbital on biscoumacetate plasma levels and prothrombin response in man

Subject	Drugs administered: Biscoumacetate Dose	Biscoumacetate Route	Heptabarbital (400 mg, oral) Pretreatment	Heptabarbital (400 mg, oral) Posttreatment	Maximum increase in prothrombin time: Whole plasma	Maximum increase in prothrombin time: 12.5% Saline diluted plasma	Plasma level of biscoumacetate after dose at: 1 hr	4 hrs	5 hrs	24 hrs
	mg		*hrs before*	*hrs after*	*sec*		*mg/L*			*mg/L*
Wi	1,800	Oral	0	0	5.8	10.2			44	<1
	1,800	Oral	1	0	1.2	0.5			4.8	<1
	1,800	Oral	0	5	3.0	15.0			66	4.6
M	1,500	Oral	0	0	16.2	68.0			170	9.3
	1,500	Oral	1	0	4.5	13.0			75	4.1
	1,500	Oral	0	5	10.0	58.0			136	9.4
S	1,500	Oral	0	0	5.5	15.0			137	4.0
	1,500	Oral	1*	0	2.0	9.0			63	3.0
	1,500	i.v.	0	0	2.0	22.5	152	83		24
	1,500	i.v.	1	0	3.0	22.0	180	110		31
Wr	1,500	i.v.	0	0	4.0	13.5	138		51	24
	1,500	i.v.	1*	0	3.5	11.0	136		47	23

* Besides the heptabarbital dose given 1 hour before the coumarin, 400 mg/day was given for 3 to 5 days before the day of the experiment.

marin and a decreased prothrombin response, compared with experiments in which the coumarin was given alone, either orally or intravenously. Administration of the barbiturate 5 hours after rather than before the coumarin, had a slight effect in some instances, and none in others. Blood levels of acenocoumarol were low (less than 3.5 mg per L) in all subjects without pretreatment,

TABLE IV

Effect of heptabarbital on Dicumarol plasma levels and prothrombin response in man

Drugs administered	Time of collection	Prothrombin times: Subject B Whole	Subject B Dil.	Subject C Whole	Subject C Dil.	Plasma conc. of Dicumarol: Subject B	Subject C
	hrs	*sec*		*sec*		*mg/L*	
Dicumarol, 500 mg p.o.	Control	13.0	29.5	14.5	33.0		
	24	13.0	35.0	15.0	51.5	23.1	29.2
	48	20.0	51.5	19.5	61.5	19.2	8.9
	72	19.5		14.5		17.1	5.7
	96					11.8	5.0
Dicumarol, 500 mg p.o.	Control	14.5	24.5	14.5	23.0		
Heptabarbital, 400 mg/day	24	15.5	27.5	15.0	33.5	14.5	1.8
for 3 days and 1 hr	48	17.0	29.5	14.0	30.0	14.1	2.2
before Dicumarol	72	16.0	30.5	14.5	27.0	6.9	3.0
	96	14.0	34.5	13.0	28.5		<0.9
Dicumarol, 500 mg p.o.	Control	13.5	26.0	15.5	29.5		
Heptabartital, 400 mg	5					21.4	8.9
5 hours after Dicumarol	24	19.5	37.5	18.5	40.0	13.6	12.5
	48	24.0	48.0	18.0	44.0	12.0	8.6
	72	20.5	43.5	15.0	31.5	10.0	4.4
Dicumarol, 500 mg i.v.	Control	13.5	32.0	13.0	31.5		
	5					35.0	63.5
	24	17.0	37.5	19.5	39.5	30.0	31.8
	48	23.0	31.0	24.5	52.0	24.0	22.6
	72	16.5		16.2		23.0	18.8
	96	17.5	33.0	17.0	35.0	18.7	11.9
Dicumarol, 500 mg i.v.	Control	13.0	22.5	14.5	23.5		
Heptabarbital, 400 mg/day	5					49.6	43.0
for 3 days and 1 hr before	24	17.0	35.0	19.5	40.0		
Dicumarol	48	23.0	43.5	22.5	50.5	17.0	19.2
	72	28.0		26.0		16.0	11.8
	96	15.5		20.5		14.0	8.7

TABLE V

Influence of heptabarbital on maximal prothrombin response to acenocoumarin in 3 human subjects

Subject*		Peak prothrombin time	
		Whole plasma	Diluted (12.5%) plasma
		sec	
E	No pretreatment	27	56
	Pretreated	22	44
F	No pretreatment	22	60
	Pretreated	19	37
H	No pretreatment	30	72
	Pretreated	25	45

* Each subject was given a single oral dose of 50 mg of acenocoumarin (no pretreatment). Three weeks later each subject received an oral dose of 400 mg of heptabarbital daily for 4 days. The last dose was followed in 1 hr by a single 50 mg oral dose of acenocoumarin.

and undetectable with pretreatment. There were individual differences in the response to coumarins, but these variations are well known from previous studies (2, 11). Pretreatment with a barbiturate had no appreciable effect on plasma levels of the anticoagulant or prothrombin time response to an *intravenous* dose of biscoumacetate.

It was noted that after an intravenous dose of biscoumacetate the plasma level at 24 hours was 20 to 30 mg per L, whereas after oral dosage the level was less than 10 mg per L (Table III). Since biscoumacetate is rapidly metabolized (about 25 per cent per hour) in man, and absorption is delayed 1 to 2 hours (11), the plasma levels of the drug 4 to 5 hours after oral dosage are consistently higher than those after intravenous administration of the same dose. Yet, the data in Table III show that at 24 hours there are higher levels following intravenous dosage than after oral dosage. The readings obtained by the method of analysis employed may represent, at least in part, a metabolite of biscoumacetate rather than the drug itself. In any event, it is apparent from these data that the metabolic fate of the drug after oral administration differs from that after intravenous administration. It is therefore conceivable that the observed difference in effect of barbiturates on orally versus intravenously administered biscoumacetate in man may be a metabolic effect rather than an effect on absorption. It has previously been shown (13) that the metabolic pathway of biscoumacetate is quite different in various species.

DISCUSSION

During studies with human subjects, Avellaneda (6) noted that a variety of orally administered barbiturates (including heptabarbital) decreased the hypo-prothrombinemic response to biscoumacetate in about two-thirds of the cases studied. The mechanism of this effect was not explained. In the present study, measurements of plasma levels of the anticoagulants revealed that barbiturate-induced reduction of hypo-prothrombinemic effect was correlated with lower plasma levels of the anticoagulant in man and in other species. The effect was observed not only with biscoumacetate but also with Dicumarol and acenocoumarin. It was shown that the relative timing of dosage and the route of administration of the coumarin derivatives were important. Barbiturate pretreatment had no appreciable influence on plasma levels or hypo-prothrombinemic effect of *intravenously* administered coumarin anticoagulants in man. Barbiturate administered *after* the coumarins also had little or no effect.

These findings exclude a vitamin K-like mechanism to explain the barbiturate effect. Unlike barbiturates, vitamin K_1 is effective even when given hours or days after the coumarin, and the action is independent of the route of administration of the coumarin; furthermore, Vitamin K does not affect the plasma levels of coumarin anticoagulants (14). Barbiturates themselves do not affect coagulation (8, 15). The barbiturates could, however, influence the physiological disposition of the coumarin. The finding that a drug can influence the disposition of another apparently unrelated drug is not unique in man (16) or experimental animals (17).

The question arises whether barbiturates affect the absorption of coumarin anticoagulants. It is known that absorption of Dicumarol is slow and sometimes incomplete (2), possibly because of the low aqueous solubility of the drug at the pH range of the gastrointestinal tract. However, acenocoumarin forms a sodium salt which is quite soluble at pH 7 (8) and biscoumacetate is rapidly absorbed from the small intestine (11).

Do barbiturates influence the rate of metabolism of the coumarin? Such an explanation may at first be considered unlikely, since in man the effect was not observed when the coumarin was

given intravenously. However, these findings in man are different from those in the guinea pig and dog. Pretreatment of guinea pigs with barbital (and also with chlorobutanol in adequate dosage) completely inhibited the hypo-prothrombinemic action of intraperitoneally administered acenocoumarin. This species difference may be related to several factors. The doses of barbiturate employed in the experiments with human subjects were small compared with those given to the guinea pigs. In the dog, pretreatment with barbiturate results in lower biscoumacetate levels after intravenous administration of the anticoagulant. Species differences in the metabolic pathway of some coumarins have been clearly demonstrated (13). The data in man suggest differences in the metabolic pathways of oral versus intravenous biscoumacetate. It is therefore possible that, in man, barbiturate pretreatment may influence the metabolic fate (other than absorption) of orally administered biscoumacetate, even though there is no detectable effect on the fate of intravenously administered biscoumacetate.

The results obtained in guinea pigs (antagonism of acenocoumarin by barbital and chlorobutanol) and dogs (antagonism of biscoumacetate by barbital) are similar to the earlier observations of Baumann, Field, Overman and Link (4, 5) (antagonism of Dicumarol by chlorobutanol, aminopyrine, and so forth) in rats. Several of the drugs that produce this antagonism, including barbiturates, were known to increase the biosynthesis, excretion and tissue levels of L-ascorbic acid in dogs and rats (18). In fact, Link and co-workers studied the effect of the "vitamin C stimulators" on Dicumarol-induced hypo-prothrombinemia, because they had previously observed that L-ascorbic acid can influence and even antagonize coumarin-induced hypo-prothrombinemia (19, 20).

Might the coumarins themselves, like the barbiturates, stimulate the enzymes responsible for coumarin metabolism? After prolonged continuous therapy with biscoumacetate, the plasma level pattern upon discontinuation is the same as that following the initial dose (3). Repeated intravenous doses of Dicumarol or biscoumacetate given to the same subject result in similar plasma level curves (2, 13). In fact, in the same subject the half-life of a large dose of Dicumarol or biscoumacetate is longer than that of a small dose (2, 11). Thus, at least in man, coumarins do not accelerate their own metabolism in therapeutic dosage.

Coumarin anticoagulant therapy is frequently accompanied by the administration of other drugs, including barbiturates. This may add to various other difficulties (1–3, 11) in controlling the hypoprothrombinemic effect of coumarins in the treatment of thromboembolic diseases. It may be necessary in the future to pay more attention to the mutual influence of concomitant therapy on the physiological disposition of the drugs employed.

SUMMARY

Results obtained in guinea pig, dog and man show that pretreatment with barbiturates may antagonize the hypo-prothrombinemic effect of coumarin anticoagulants. This effect has been correlated with lower plasma levels of the anticoagulant drugs. In man, route and timing of dosage significantly influence this barbiturate effect.

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PREPARATION AND PURIFICATION OF HUMAN INSULIN-I^{131}; BINDING TO HUMAN INSULIN-BINDING ANTIBODIES

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(Submitted for publication April 10, 1961; accepted June 2, 1961)

Indirect evidence has been adduced that insulin-binding antibodies in man, formed in response to treatment with commercial mixtures of beef-pork insulin, react also with human insulin (1). Thus, human insulin inhibits competitively the binding of I^{131}-labeled beef, pork, sheep or horse insulin to antibeef-pork insulin antibodies. However, the observation that much higher concentrations of human insulin than of animal insulins may be required to effect a comparable reduction in the binding of animal insulin-I^{131} suggests that the reaction with human insulin is, in such cases, considerably weaker than with animal insulins (1). The failure to detect reaction with human insulin in some studies (2) may be interpreted (1) on this basis. It also appears likely that these quantitative relationships may explain why certain diabetic subjects can exhibit a moderate degree of resistance to animal insulins and yet maintain fairly good control of blood sugar through stimulation of their own endogenous insulin secretion in the absence of exogenously administered insulin (3).

In order to study the reaction of human insulin with antibody in a direct manner, the use of I^{131}-labeled human insulin is mandatory. Since pure crystalline human insulin is not generally available,[1] a crude preparation must serve as starting material. The purposes of this communication are to describe the preparation and purification of human insulin-I^{131}, to demonstrate its reaction with human antibodies against beef-pork insulin and to consider the clinical implications of such reaction.

METHODS

All serums were taken at least 24 hours after the last dose of insulin and were allowed to remain at 4° C for at least 2 months to permit destruction by plasma (3) of any residual insulin. Two patients (H.L. and M.N.) had not received insulin for about 1 year and 4 years, respectively, prior to withdrawal of the serum tested.

The starting material employed for iodination was a powdery preparation of human insulin generously supplied by Dr. A. M. Fisher, Connaught Laboratories, Toronto, which assayed at 6.8 U per mg in March 1956 (4), but was thought possibly to have decreased slightly in hormonal potency by 1959. Since several crystalline animal insulins obtained from Dr. Fisher were estimated at about 22 U per mg, we had tentatively assigned a value of 6.0 U per mg to the human insulin based on a reference standard of 22 U per mg for *pure* insulin (5). This material was tested by immunoassay in 1959 against a preparation of human insulin, obtained from Dr. F. Tietze (through the courtesy of Dr. J. Field, National Institutes of Health), which had recently been assayed in the Lilly Research Laboratories. The assigned value of 6.0 U per mg for the Fisher insulin referred to a standard of 22 U per mg for pure insulin agreed excellently with the recently standardized Tietze insulin and was therefore accepted as essentially correct and indicating an insulin content of 6.0 U per mg/22 U per mg = 27.3 per cent insulin by weight (5). More recently we have assayed the Fisher insulin against a preparation of *pure* human insulin[2] supplied through Dr. Ellis Samols, London, by courtesy of Prof. F. G. Young, Cambridge. The assigned value of 27.3 per cent insulin by weight for the Fisher preparation was found to agree excellently with the Cambridge pure insulin, in confirmation of the results recently obtained by Dr. Samols (6) who found the Fisher crude powder to be "consistently equivalent to 25 to 30 per cent Cambridge insulin by weight." The Fisher powder was dissolved in distilled water brought to pH 2.3 with HCl to a final concentration of 200 μg per ml; 2 to 4 vol of 0.2 M borate buffer, pH 8.0, was added and 50 μg of the material was iodinated with I^{131} by methods described previously (5). Carrier-I^{127} was added in amounts not exceeding a ratio of 0.2 iodine atom per insulin monomer (mol wt 6,000) in the final labeled mixture. After dialysis against distilled water at 4° C to remove unbound iodine, human serum albumin was added to a concentration of 2 per cent and the solutions kept frozen until used.

All preparations and fractions obtained during the purification procedure were analyzed by paper chromatography[3] and electrophoresis. It has previously been

[1] To our knowledge, human insulin has not yet been obtained in crystalline form.

[2] Purified on cellulose ion-exchange columns by the method of Dr. J. F. Smith, Cambridge, England (6).

[3] During this procedure, the strips are also subjected to electrophoresis, but the principal separation of pro-

TABLE I

Purification of I^{131}-labeled human insulin on cellulose column

	Radioactivity
	%
Original effluent + 1st veronal wash	71.0
2nd veronal wash	4.4
3rd veronal wash	1.3
4th veronal wash	.7
1st plasma eluate (2 ml plasma)	9.7
2nd plasma eluate (1 ml plasma)	3.7
Remaining on cellulose column	9.3

demonstrated that undamaged crystalline animal I^{131}-insulins in plasma adsorb to Whatman 3 MM paper at the site of application (7, 8), whereas damaged fractions (8) and antibody-bound insulin (8) migrate with the serum proteins. It can therefore be accepted that any I^{131}-labeled material which migrates with the serum proteins of nonimmune plasma is definitely not undamaged insulin-I^{131}, whereas any labeled fraction that is adsorbed to paper under these conditions *might be* insulin-I^{131}. The final criterion required to establish that I^{131}-labeled material adsorbed to paper in the presence of control plasma is, in fact, insulin-I^{131} is the demonstration of its binding to antibody in antiserums from animals (or man) immunized with animal insulins of high purity. Only that fraction of adsorbed radioactivity which is demonstrably reactive with insulin-binding antibodies can be considered unequivocally to represent insulin-I^{131}.

The cellulose adsorption characteristics of insulin were exploited in the purification procedure. Powdered cellulose (Whatman) columns approximately 3.5 cm (ht) × 0.5 cm (diam) were packed tightly and moistened with veronal buffer (ionic strength 0.1, pH 8.6). Aliquots of I^{131}-labeled crude human insulin were added to 100 μl control human plasma and passed through the column. The original effluent and several washes with veronal buffer were monitored for radioactivity; when radioactivity in the wash had been reduced to a negligible level, the residual adsorbed I^{131}-labeled material was eluted with several aliquots of whole control plasma, previously treated with iodoacetamide as described below.

The principle of separation of insulin-I^{131} from other I^{131}-labeled compounds in the mixture depends on the capacity of the cellulose to adsorb small amounts of insulin preferentially in the presence of limited amounts of plasma and the ability of high concentrations of plasma to elute the adsorbed insulin-I^{131}.[4] Since the insulin-adsorbing capacity of the cellulose is limited, use of large amounts of insulin results in a considerable fraction being lost with the original effluent. If the total amount passed through the column does not exceed 1 to 2 μg, approximately 20 to 50 per cent or more is adsorbed by the column, whereas contaminants and damaged fractions which bind to or migrate with plasma proteins on paper strips pass through the column with the original effluent and veronal washes. One ml plasma will generally elute about 20 to 30 per cent of the adsorbed radioactivity. However, plasma itself, especially at 37° C and after extended periods of incubation even at 4° C, can damage insulin (8). Since the chromatographic and electrophoretic characteristics of such damaged insulin resemble, in many respects, insulin altered by cysteine or irradiation (9), both of which are known to effect reductive splitting of disulfide bonds (10, 11), it seemed possible that free sulfhydryl groups in plasma (sulfhydryl groups of serum proteins, free cysteine, reduced glutathione, and so forth) might be responsible in part for "plasma damage" to insulin-I^{131}. This hypothesis was tested by treating control plasma with 0.5 per cent iodoacetate or iodoacetamide (30 minutes at 37° C) prior to incubation with insulin-I^{131}. This treatment was effective to a large extent in protecting crystalline beef insulin-I^{131} against damage by plasma.[5] Accordingly, plasma used for elution of insulin-I^{131} was first treated with iodoacetamide as indicated.

RESULTS

A typical purification experiment is summarized in Table I. Scans of radioactivity on paper chromatoelectrophoretograms and electrophoretograms of the unpurified solution, the original effluent and plasma eluates 1 and 2 are shown in Figure 1. In all preparations (13 separate iodination procedures) the fraction of radioactivity in the unpurified solutions that migrated with the serum proteins on chromatography was about 35 ± 5 per cent of the total protein-bound I^{131}. On electrophoresis (Figure 1), the migrating radioactivity is seen to be randomly distributed throughout the zone of migration of serum proteins, but there is, in addition, a small fraction extending out beyond serum albumin.

The original effluents contained, as expected, a greater proportion of migrating activity as well

teins from the site of application is a result of hydrodynamic flow chromatography rather than electrophoresis (8).

[4] This phenomenon has a counterpart in paper strip chromatography or electrophoresis. If the paper strip is first *soaked* in plasma, insulin-I^{131} will be adsorbed only slightly or not at all.

[5] Prolonged incubation of plasma containing 0.05 to 0.5 per cent iodoacetate with insulin-I^{131} for periods longer than 6 to 8 hours at 37° C results in loss of ability of insulin-I^{131} to adsorb to cellulose, possibly a result of slow interaction of iodoacetate with amino groups as is suggested by an increase in electrophoretic mobility of all the serum proteins. (Incubation at 4° C for as long as 9 days does not generally lead to this change.) However, the ability of the altered insulin-I^{131} to bind to antibody is not detectably impaired.

as virtually all the iodide^{131} not removed by dialysis. Adsorbed radioactivity eluted with plasma contained 2 to 6 per cent migrating components. Evidence that the fraction remaining at the origin (in control plasma) is largely, if not entirely, insulin-I^{131} is provided by experiments with specific insulin antiserums. When tracer quantities of the purified I^{131}-labeled human insulin were incubated with guinea pig antibeef insulin or antipork insulin serums or with human antibeef-pork insulin serums containing high concentrations of insulin-binding antibodies, at least 80 to 90 per cent of the adsorbed radioactivity was bound to antibody (Figure 2). This is true also of the adsorbed radioactivity in the unpurified preparations. It is possible that even higher fractions were bound to antibody in the test tubes and that dissociation of the insulin-antibody complexes during migration resulted in the observed trailing of 10 to 20 per cent. In support of this suggestion is the observation that the percentage remaining at the origin and trailing up to the antibody region

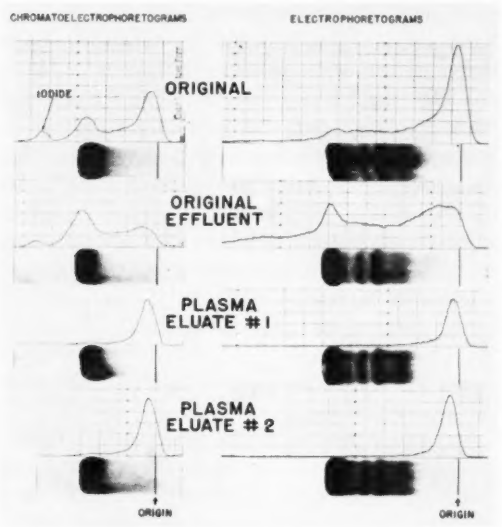

FIG. 1. RADIOACTIVE SCANS OF PAPER STRIPS AFTER CHROMATOELECTROPHORESIS AND CONVENTIONAL ELECTROPHORESIS OF CRUDE HUMAN INSULIN-I^{131} ("ORIGINAL") AND FRACTIONS OBTAINED DURING PURIFICATION ON A CELLULOSE COLUMN. All fractions were added to control (nonimmune) plasma prior to application on the paper strips. Chromatoelectrophoresis requires about 30 to 60 minutes, resolves the iodide peak, and lumps most non-insulin-I^{131} components into a single peak moving away from the origin with the serum proteins.

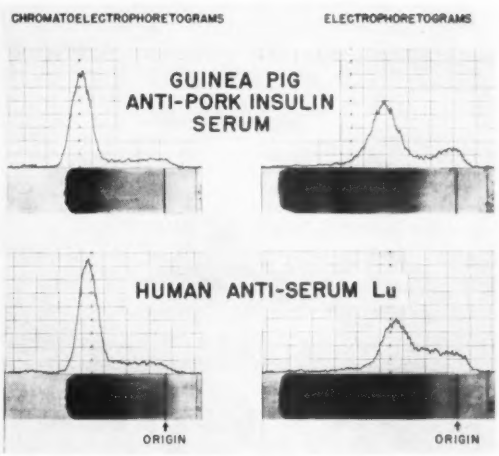

FIG. 2. RADIOACTIVE SCANS OF PAPER STRIPS AFTER CHROMATOELECTROPHORESIS AND CONVENTIONAL ELECTROPHORESIS OF HUMAN INSULIN-I^{131} (PLASMA ELUATE NO. 1 OF FIGURE 1) ADDED TO INSULIN ANTISERUMS. Note that insulin-antibody complex migrates between γ- and β-globulins in both guinea pig and human antiserums, whereas only negligible amounts of radioactivity migrate with serum proteins in control (nonimmune) serums (Figure 1).

is greater in the electrophoretograms run over an 18 hour period than in the short-run (30 to 60 minutes) chromatoelectrophoretograms, the longer time required in the former procedure allowing for greater dissociation. On the other hand, in similar experiments with beef insulin-I^{131}, 99 per cent or more of the radioactivity is demonstrably bound to antibody in both chromatoelectrophoretograms and electrophoretograms.

Since generally about 65 per cent of total protein-bound-I^{131} in the unpurified solution is adsorbed by paper in the presence of control serum, these results indicate that approximately 50 to 60 per cent of the protein-bound-I^{131} in the unpurified preparation represents insulin-I^{131}. Since insulin comprises only 27 per cent by weight of the preparation used for iodination, it can be concluded that one-half or more of the non-insulin contaminants is either not able to be iodinated under these conditions, is iodinated less readily than insulin itself, or is dialyzable.

To evaluate quantitative differences between the reaction of human and beef insulin with antibody, antiserums from eight insulin-treated diabetic subjects were incubated for 15 minutes at 37° C with mixtures of human insulin-I^{131} and

TABLE II

Insulin-binding capacity of serum

Subject	Insulin dosage at time serum drawn	Maximum beef insulin-binding capacity of serum*	Insulin concentration tested	Insulin bound after 15 min at 37°C†	
				Human	Beef
	U/day	*U/L*	*U/L*	*U/L*	*U/L*
M.N.	0	7	0.25	0.10	0.17
			1.0	0.41	0.66
Mo	50	5.5	1.0	0.71	0.70
Low	65	7	0.25	0.14	0.19
			1.0	0.52	0.74
H.L.	0	22	3.0	0.56	1.6
Gal	90–110	28	0.25	0.21	0.23
			1.0	0.81	0.88
Lo	Unknown, but known to be resistant	97	0.25	0.20	0.24
			1.0	0.72	0.93
Gru	800	210	0.25	0.18	0.23
			1.0	0.74	0.89
Ge	700	250	0.25	0.19	0.23
			1.0	0.71	0.94
			3.0	1.7	2.6

* Determined with diluted antiserum and higher insulin concentrations at equilibrium (after prolonged incubation).
† Corrected for damaged insulin-I^{131} and assuming that only 85% of adsorbed human insulin-I^{131} was available for binding to antibody.

unlabeled Fisher human insulin or beef insulin-I^{131} and unlabeled beef insulin at total insulin concentrations of 0.25, 1.0, or 3.0 U per L, and then applied to paper strips in a cold room (4° C) for paper chromatoelectrophoresis. Of the eight antiserums studied (Table II), three (Lo, Gru, Ge) were from patients who were quite definitely insulin-resistant and had insulin-binding capacities ranging from 97 to 250 U per L; one was from a patient (Gal) who could probably be termed mildly resistant and had a binding capacity of 28 U per L, and one was from a subject (H.L.) reported previously (3) who had an insulin-binding capacity of 22 U per L. The lowest of the concentrations used (0.25 U per L) is in the range of values of endogenous insulin found in normal subjects and in patients with early maturity-onset diabetes, 0.5 to 2 hours after oral administration of 100 g glucose (5); the second concentration used (1 U per L) is at the upper limits of the range of values observed after heavier glucose loading. The highest concentration employed (3 U per L) is considerably beyond the range of endogenous insulin concentrations that we have observed in man (5), but is certainly frequently exceeded in insulin-resistant subjects given large therapeutic doses of insulin.

In almost all cases, more beef insulin than human insulin was bound at the same insulin concentration by the same antiserum (Figure 3, Table II) but in some cases the differences were small after correction for the fraction of radioactivity presumed unavailable for binding to antibody. However, in several subjects (M.N., H.L. and Ge) the binding of human insulin was significantly lower than the binding of beef insulin at the same concentration. In all subjects the concentrations employed were generally far below the maximal binding capacities of the antiserums so that frequently most of the insulin was bound. At higher concentrations more marked differences might have become evident.

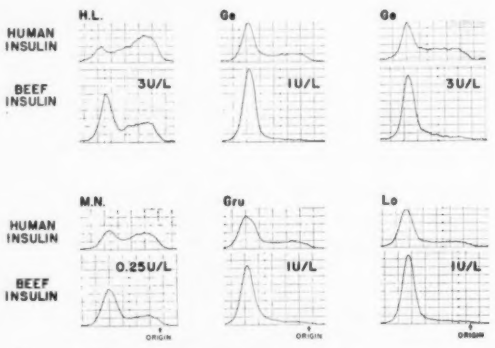

FIG. 3. RADIOACTIVE SCANS OF PAPER STRIPS AFTER CHROMATOELECTROPHORESIS OF INSULIN-ANTISERUM MIXTURES INCUBATED FOR 15 MINUTES AT 37° C.

DISCUSSION

Studies such as have been reported here should become considerably easier to perform when pure human insulin becomes available. However, it is evident that the crude preparation employed is adequate for the demonstration of the binding of human insulin to human antibeef-pork insulin antibodies and for general quantitative estimation of the extent of such binding. In previous studies on the competitive inhibition offered by unlabeled human insulin to the binding of I^{131}-labeled animal insulins (1), only a weak inhibitory effect of human insulin was observed with antiserum H.L. In the present study this finding is confirmed by the direct demonstration of a weak reaction with human insulin-I^{131}, but such marked differences between human insulin and beef insulin are seen not to hold uniformly in all subjects. In other experiments, not reported here, in which unlabeled human insulin and unlabeled beef insulin each competed with beef insulin-I^{131}, it was observed that the competitive reaction with human insulin is strong where the direct reaction of human insulin-I^{131} is strong and that a weak competitive reaction is confirmed by a weak direct reaction, as in Subject H.L.

The differences in behavior of various antiserums toward human and beef insulin help explain certain clinical phenomena. For example, Subject H.L., whose clinical course is described in greater detail elsewhere (3), maintained essentially the same moderately good control of his diabetes in the absence or presence of insulin therapy (55 U protamine zinc insulin daily). It can be concluded that the strong binding of beef insulin to his antibodies deprived him of the benefit of this treatment, whereas the much weaker binding of human insulin permitted the advantageous utilization of his own endogenously secreted human insulin. Also, Subject M.N., who took insulin therapy for about 1 year but who has for the past 4 years maintained excellent control without insulin treatment, showed a stronger reaction with beef insulin than with human insulin, although his insulin-binding capacity was in the range found in nonresistant subjects. Furthermore, experience has shown that some insulin-resistant patients may be well controlled on sulfonylurea drugs. Presumably the endogenous insulin secreted in response to stimulation by the sulfonylureas reacts less well with antibody than do the administered animal insulins. Another case in point is the report of Lowell (12) that 30 U of human insulin was capable of reducing the blood sugar in a patient resistant to a similar dose of beef-pork insulin.

The reaction of human insulin with human antiserums to beef-pork insulin does not, of course, imply that human insulin is antigenic in man. In fact, we have never observed any evidence of binding of insulin in serums of patients who never received animal insulin. The reaction simply indicates that once the antigenicity of animal insulins has resulted in the production of antibodies, these antibodies are then able to react also with the closely similar human insulin. Since insulin-antibody complexes are of small molecular size, remain soluble (3), and do not appear to be sensitizing, allergic manifestations of such complex formation are not exhibited.[6] Such might not be the case were the antigenic characteristics of insulin somewhat different. It appears not beyond the realm of possibility that certain autoimmune states may originate by immunization with foreign antigens (through ingestion or by entry through other portals) which chemically resemble certain endogenous proteins.

SUMMARY AND CONCLUSIONS

1. The preparation of I^{131}-labeled human insulin from a lot of human insulin containing approximately 25 per cent insulin by weight and its purification from labeled contaminants are described.

2. The reaction of human insulin-I^{131} with insulin-binding antibodies in the serums of human subjects treated with commercial mixtures of animal insulins is demonstrated directly.

3. Comparison of the binding of human insulin and beef insulin in antiserums from eight insulin-resistant and nonresistant diabetic subjects revealed a lesser affinity of antibody for human than for beef insulin in most cases, but considerable variability in this respect was encountered among different antiserums.

[6] Allergy to insulin is an expression of reaction with sensitizing antibody (13), an antibody quite distinct from "blocking" antibody (13) and the insulin-binding antibody reported here (14).

4. It is suggested that the ability of certain diabetic patients (who show evidence of insulin resistance to exogenous insulin) to tolerate withdrawal of insulin therapy, with or without the aid of sulfonylurea agents, is related to the relatively weak binding to antibody of endogenously secreted human insulin.

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SKELETAL DYNAMICS IN MAN MEASURED BY NONRADIOACTIVE STRONTIUM *

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A great deal of useful information about calcium metabolism in man has been obtained by balance technics. Balance studies give only net changes in total body calcium and do not indicate whether changes in balance result from alterations of rates of movement into or out of bone. For example, positive balance could result from increased bone deposition or decreased bone resorption. Similarly, balance could be unchanged if increased resorption were matched by increased deposition. Rates of bone deposition have been measured in man using Ca^{45} and Ca^{47} as tracers (1–11). Isotopes of strontium have been similarly used (5, 6, 8, 11–16), since strontium is a bone-seeking element whose distribution in the body is similar to that of calcium. Accumulated experience suggests that cautious use of these bone-seeking radionuclides in human subjects is without demonstrable risk. Nevertheless, this experience is still limited both in numbers of subjects and length of observation. In a study of Sr^{90} toxicity in mice, a direct linear relationship was noted between total internal radiation dose from Sr^{90} and decrease in survival time and tumor production (17). Even small doses of radiation may not be without danger, for this analysis showed no lower threshold.

A recently developed flame photometric method (18) for measurement of calcium was adapted to measure strontium. If stable strontium could be used as a tracer it would be valuable for safely studying various types of patients in statistically valid numbers and for repeated tests in the same individual under varied conditions. This possibility is the subject of the present investigation. The results indicate that nonradioactive strontium can be used to detect alterations of skeletal metabolism in various defined physiological and pathological states.

METHODS

The following subjects were studied: 25 healthy persons at ordinary activity (including 11 inmates of San Quentin Prison; 31 studies); 14 athletes (all members of the San Quentin Prison football team); 11 patients with idiopathic nephrolithiasis; 28 with primary hyperparathyroidism (4 with skeletal disease shown by elevated serum alkaline phosphatase levels or roentgenographic evidence of skeletal involvement or both; 17 with normal phosphatase and X-ray appearance but with microscopic foci of resorption seen in bone biopsy; and 7 with normal phosphatase, X-ray appearance and bone biopsy); 7 with hyperthyroidism; 8 with acromegaly; 3 with hyperadrenocorticism; 26 with advanced, unequivocal postmenopausal or senile osteoporosis (40 studies); 5 with Paget's disease of bone; and 1 with chronic vitamin D overdose. The reproducibility of the method was tested by 21 duplicate tests in osteoporotic and normal subjects. All subjects were on diets free of milk and cheese except the prisoners, whose fare was unrestricted. All subjects were ambulatory.

Significant dietary strontium was excluded by checking initial blood and urine specimens. Then 10.0 mEq of strontium gluconate [1] was infused intravenously over a period of 10 minutes. Blood samples were taken every 24 hours and 24-hour urine specimens were collected for 4 to 6 days.

All specimens were collected in acid-washed glassware. All determinations were done in duplicate. Serum and urine calcium levels were determined on a flame spectrophotometer by the method of MacIntyre (18). Serum strontium was measured by diluting 1.0 ml of serum with 3 ml of an aqueous solution of 3 per cent perchloric acid and 0.5 mM monopotassium phosphate. The supernatant was read at 461 mμ in the flame spectrophotometer between standards in a 0.25 dilution of serum containing concentrations of sodium, potassium, and calcium which

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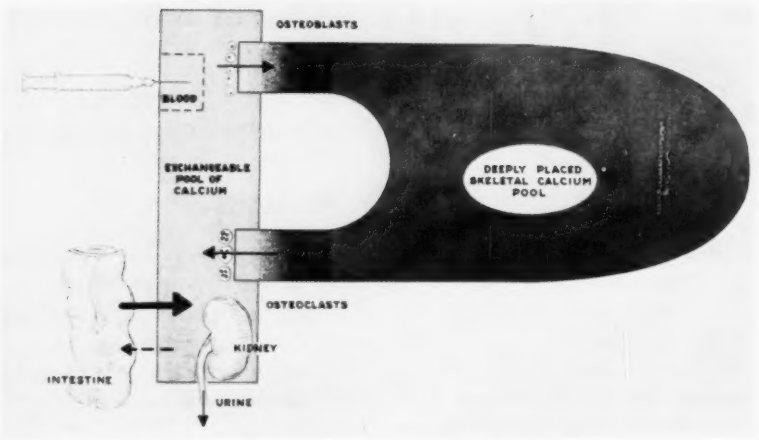

FIG. 1. MODEL FOR ANALYSIS OF SKELETAL DYNAMICS.

are in the plateau area of their effect in enhancing strontium emission (19). Complete recovery of urinary strontium required co-precipitation with calcium as follows. Three ml of saturated ammonium oxalate was added to 15 to 30 ml of urine, pH was adjusted to between 5.5 and 6.0; then 0.25 ml of 10 per cent calcium gluconate was added and the urine heated in a boiling water bath for a few minutes until the precipitate became flocculent. The urine was cooled at 4° C for 2 or more hours, then centrifuged at 2,500 rpm for 10 minutes. After the supernatant was decanted, the precipitate was dissolved in 5.0 ml of 2 N hydrochloric acid and made up to a total volume of 15.0 ml with distilled water. This solution was analyzed in the flame spectrophotometer, always by reading between adjacent standards. When this precipitation technic was checked with radioactive strontium tracer, recovery varied from 97 to 101 per cent.

TABLE I

Strontium/calcium observed ratios between serum and body tissues and fluids after intravenous infusion of strontium gluconate

Time after infusion		Source	$\frac{Sr}{Ca}_{source}/\frac{Sr}{Ca}_{serum}$
hrs	*min*		
9	5	Ascitic fluid	0.99
9	5	Ascitic fluid	0.89
9	5	Ascitic fluid	0.97
20	30	Ascitic fluid	0.94
21	0	Ascitic fluid	0.90
22	45	Ascitic fluid	0.82
22	45	Ascitic fluid	0.94
22	45	Ascitic fluid	0.84
32	15	Ascitic fluid	0.73
32	15	Ascitic fluid	0.81
32	15	Ascitic fluid	1.02
40	0	Spinal fluid	1.04
4	20	Gall bladder	0.47
4	20	Breast fat	0.48
4	35	Rectus abdominis	0.44
5	0	Strap muscle	0.49
14	35	Strap muscle	0.60
20	0	Strap muscle	0.45

Analysis of data. The model for analysis of calcium movement is similar to that proposed by others (4, 7, 14, 15). This model (Figure 1) is based on studies which suggest that body calcium is divided into two pools (1, 3, 20–22). One is a very small pool of rapidly exchangeable calcium in the extracellular fluid, soft tissues, and surface crystal of bone. The other is a very large pool of less readily available calcium in the deeper layers of bone. Calcium is added to the exchangeable pool by intestinal absorption and bone resorption. It leaves the exchangeable pool by excretion and bone deposition. Thus, if one can introduce a tracer into the exchangeable pool and then measure the rate of loss of tracer from the pool and the rate of excretion, one can calculate the rate of bone deposition as the difference between the two. Such analysis can be done by using the usual formulas for measuring pool size and pool turnover rates. The calculations shown require the assumptions that there be no change in pool size, that mixing in the exchangeable pool is complete (or almost so) at the time that observations used for the calculations are made, that there is no continuing concentration (sequestration) of tracer in tissues other than bone during the period of observation, and that there is no return of tracer to the pool from bone.

Important alterations in the size of the exchangeable pool are not likely in 4 to 6 days and could not occur imperceptibly because such an alteration requires excessive diuresis or loss of soft tissue or skeleton. (No such fluctuation in body weight, urine volume, or urinary calcium excretion occurred during these studies.) That mixing of strontium through the various compartments of the exchangeable pool actually occurs within 24 hours after injection was tested in a few patients by comparing Sr to Ca ratios of available fluids and tissues with Sr to

Ca ratio of serum at varying time intervals after injection (Table I). These figures confirm that intravenously injected strontium is largely equilibrated through body fluids and soft tissues by 24 hours, as is calcium (20, 23–25). The slightly longer mixing time indicated by the serum curves suggests that skeletal equilibration is a little slower. This is borne out by external counting over soft tissue and bone (8). After distribution of intravenously injected calcium and strontium, most of the retained dose moves into the skeleton, and there is no continued accumulation in other tissues (8, 23, 26, 27). Continued concentration of tracer in tissue other than bone would add an additional route of tracer loss from the pool. The studies of Heaney and Whedon (7) indicate that in most subjects tracer does not re-enter the pool until 6 to 8 days after its injection, and it may be assumed that bone growth and bone resorption do not occur simultaneously in the same sites. Hence the major assumptions for the analysis seem reasonable. Accordingly, serum strontium concentrations were plotted as in Figure 2 and the following calculations were made:

$$k = 0.693/t_{\frac{1}{2}} \qquad [1]$$

where k = fractional rate constant and $t_{\frac{1}{2}}$ = "half-time" derived by plotting serum specific activities.

$$E = D/s_0 \qquad [2]$$

where E = exchangeable pool (volume of strontium distribution), D = corrected dose of tracer and s_0 = serum specific activity at time zero.

$$T = kE \qquad [3]$$

where T = total turnover rate.

$$U = \int_{t_1}^{t_2} dU \; s_0 \int_{t_1}^{t_2} e^{-kt} dt = (U_{t_2-t_1})k/(s_{t_1} - s_{t_2}) \qquad [4]$$

where U = urinary excretion rate, $U_{t_2-t_1}$ = urinary content of tracer between time 1 and time 2 (usually the interval from 24 to 120 hours) and s_{t_1}, s_{t_2} = serum strontium concentration at time 1 and time 2.

$$B = T - U \qquad [5]$$

where B = rate of bone deposition.

During the 24 hours required for mixing, the strontium concentration in some parts of the pool will be greater than in others. Since the high concentration areas include blood, renal excretion will be temporarily enhanced. The excess excretion due to this inequality in distribution during mixing can be corrected by subtracting theoretical from actual urinary strontium measured during this interval. The theoretical urinary excretion during the mixing period was calculated from Equation 4 by letting t_1 and t_2 be the times at the beginning and end of mixing. The amount of excess excreted was usually 5 to 15 per cent of the dose. The injected dose was corrected for this amount in calculation of pool size. Examination of serum strontium curves indicated 24 hours as an average mixing time, with individual variations between 20 and 30 hours. The error introduced by using the initial 24 hours as a standard mixing time is small, since the steep portion of the mixing curve was always included in the correction.

To make the method widely applicable clinically, two routes of tracer excretion were not usually measured—sweat and feces. While some authors (28, 29) have suggested that sweat is normally an important route of calcium loss, our measurements of sweat calcium and strontium concentrations during physical exercise (Table II) showed that even at maximal rates, sweat accounted for only a small portion of calcium or strontium loss. The sweat glands, like the intestine and kidney, discriminate against strontium. Fecal losses were not measured

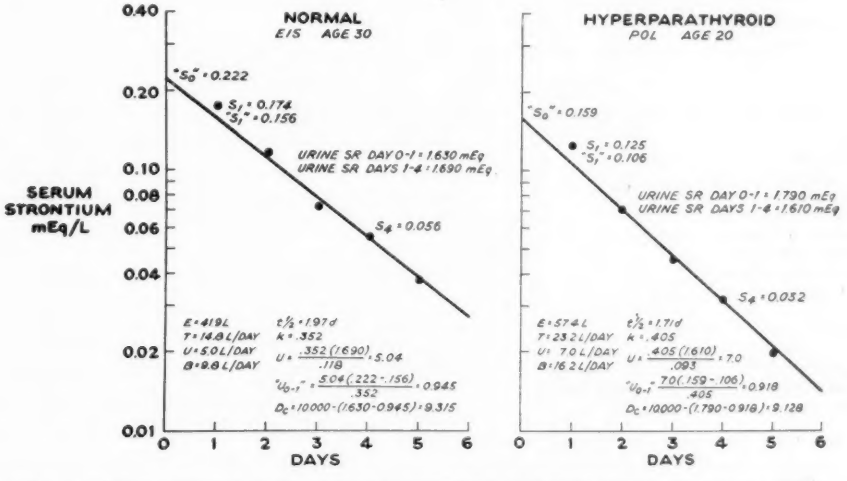

FIG. 2. REPRESENTATIVE PLOTS OF SERUM STRONTIUM CONCENTRATIONS AND CALCULATIONS OF SKELETAL DYNAMICS IN A NORMAL AND A HYPERPARATHYROID SUBJECT.

TABLE II

Sweat calcium and strontium content

Name	Period*	Serum Ca	Serum Sr	Serum Sr/Ca	Sweat Ca	Sweat Sr	Sweat Sr/Ca
		mEq/L			*mEq/L*		
EIS	1	5.40	0.243	0.045	1.86	0.103	0.055
	2		0.235		1.24	0.114	0.092
	3	5.65	0.216	0.038	1.78	0.089	0.050
WHI	1	5.65	0.216	0.038	1.75	0.085	0.049
	2		0.206		1.44	0.063	0.044
	3	6.10	0.197	0.032	1.83	0.071	0.039
LUB	1	5.35	0.215	0.040	1.92	0.110	0.057
	2		0.197		0.99	0.085	0.086
	3	6.10	0.178	0.029	1.48	0.105	0.071

* Periods 1, 2, and 3 are three consecutive 30-minute periods commencing 16 hours after infusion of strontium and during which subjects exercised. Sweat was collected on covered patches of ash-free filter paper from previously washed areas of skin. Filter paper was eluted with 2 N HCl. Calcium and strontium were determined flame spectrophotometrically as for urine.

in our routine studies. Harrison, Raymond and Tretheway (12) reported 5-day cumulative fecal excretion of 3 per cent of an intravenous dose of strontium. Spencer and her co-workers (13) reported approximately 9 per cent in 6 days (1 patient) and from 8 to 17 per cent in 12 days (7 patients) (30), and concluded that the major route of excretion is through the kidney. Dow and Stanbury (11) found from 2.5 to 19 per cent fecal excretion of Sr^{85} in 6 days in various disease states. Fraser, Harrison and Ibbertson (15) found that fecal excretion of intravenously injected stable strontium is 8 to 16 per cent of the administered dose in 6 days in the absence of diarrhea or steatorrhea. Calculation of the portion of pool lost daily through fecal excretion by our subjects based on the published range of values for fecal excretion shows that bone deposition is overestimated by 9 to 16 per cent in all groups by ignoring fecal excretion. This discrepancy does not appear to affect comparison between our groups but will affect, by this amount, comparison with data in which fecal excretion has been measured.

RESULTS

The size of the exchangeable strontium pool in 25 healthy subjects at ordinary activity was 42.7 L, of which 30.9 per cent was turning over daily (Table III).[2] Urinary excretion from the pool as measured by strontium was 3.9 L per 24 hours, and the rate of deposition in bone was 9.6 L per 24 hours. In 11 patients with a history of urinary tract stones but without hypercalcemia, the pool size was comparable to that of the control subjects, but more mineral went to urine and less to bone. In the 14 athletes the pool was larger, and 35.7 per cent was turning over daily. The rates of urinary excretion and deposition in bone were 50 per cent greater than in healthy people at normal activity.

Hyperparathyroidism with X-ray or phosphatase evidence of osteitis also enlarged the pool, and both urinary excretion and bone deposition were increased. When only microscopic evidence of osteitis could be found the pool was only very

[2] The individual data from which Table III has been compiled appear in Tables I through IX in the Appendix.

TABLE III

Strontium dynamics in various conditions *

Condition	No. of studies	No. of subjects	k	E	T	U	B
				L	*L/24 hrs*	*L/24 hrs*	*L/24 hrs*
Healthy; ordinary activity	31	25	0.309±0.010	42.7± 1.1	13.5± 0.6	3.9±0.2	9.6±0.4
Athletes	14	14	0.357±0.014	56.9± 2.3	20.7± 1.0	5.8±0.2	15.0±1.0
Nephrolithiasis	11	11	0.340±0.017	39.3± 2.3	13.3± 0.9	5.7±0.8	7.6±0.6
Hyperparathyroidism:							
with X-ray osteitis or increased alk. phosphatase;	4†	4	0.407 (0.269–0.517)	64.4 (50.8–73.7)	26.2 (18.4–33.6)	7.3 (4.7–8.8)	18.9 (12.8–24.8)
normal X-ray appearance and alk. phosphatase, microscopic osteitis on iliac crest biopsy;	18	17	0.396±0.025	49.1± 2.8	18.5± 0.8	6.9±0.5	11.5±0.6
no evidence of osteitis by X-ray exam., phosphatase, or bone biopsy	7	7	0.309±0.023	38.8± 3.2	11.8± 1.1	4.3±0.5	7.5±0.9
Hyperthyroidism	7	7	0.475±0.051	48.9± 3.6	23.8± 3.5	7.0±2.2	16.7±2.0
Acromegaly	9	8	0.332±0.022	73.8± 6.2	23.7±2.6	5.7±0.5	18.2±2.3
Osteoporosis, postmenopausal	40	26	0.263±0.007	33.0± 0.9	8.7± 0.3	2.3±0.2	6.4±0.3
Adrenocortical hyperplasia	2‡	2	0.413–0.537	19.8–28.2	8.2–15.2	5.8–8.6	2.4–6.6
Paget's disease	5†	5	0.508 (0.336–1.040)	95.4 (41.5–175.5)	58.7 (16.4–182.5)	4.7 (2.3–6.8)	54.0 (14.1–175.7)
Chronic vitamin D overdose	1	1	0.330	376.0	124	0.5	123.5

* k = Serum disappearance rate constant, E = exchangeable pool, T = total turnover, U = urinary excretion, B = bone deposition. Values are given as mean ± standard error.

† Mean and range.

‡ Individual values of 2 subjects are given.

TABLE IV

Strontium dynamics, before and after cure of hyperparathyroidism *

Patients	Status	Serum Ca	k	E	T	U	B
		mEq/L		*L*	*L/24 hrs*	*L/24 hrs*	*L/24 hrs*
NOR	Pre-op.	7.00	0.359	49.9	17.9	6.3	11.6
	4 days post-op.	4.85	0.328	51.0	16.7	5.9	10.8
POL	Pre-op.	7.35	0.502	58.2	29.2	10.0	19.2
	3 mos after 1st test	7.05	0.405	57.4	23.2	7.0	16.2
	1 week post-op.	5.26	0.563	43.2	24.3	2.8	21.5
TUR	Pre-op.	6.15	0.389	43.4	16.9	5.8	11.1
	9 mos post-op.	5.55	0.269	46.0	12.4	2.9	9.5
LAM	Pre-op.	6.10	0.359	31.7	11.4	5.5	5.9
	14 mos post-op.	5.50	0.225	44.4	10.0	2.6	7.4
SWE	Pre-op.	7.30	0.245	35.8	8.8	2.7	6.1
	2 mos post-op.	4.75	0.213	35.7	7.6	0.7	6.9
	8 mos post-op.	5.20	0.259	29.3	7.6	1.4	6.2

* For abbreviations, see Table III.

slightly enlarged, and there was a similar small increase in bone deposition rate ($p < 0.01$). When even the histological appearance of the bone biopsy specimen was normal, the exchangeable pool size and urinary excretion were normal and the bone deposition rate tended to be low. In five patients without clinical evidence of osteitis fibrosa, cure of hyperparathyroidism did not change bone deposition rates, but urinary excretion rates fell in four and exchangeable pool size decreased in two (Table IV).

Calcium dynamics in the four conditions associated with osteopenia differed widely. In senile osteoporosis the mean values for exchangeable pool size and bone deposition rate were significantly less (-23 and -33 per cent, respectively) than in the normal group, although there was some overlap (Figure 3). After correction for body weight or surface area, exchangeable pool and bone deposition rates were still significantly lower in osteoporotics than in normal subjects (Table V). The two patients with osteoporosis due to long-standing Cushing's disease (bilateral adrenal cortical hyperplasia) had the smallest miscible pools in the series. A large portion of the pool was excreted daily by the kidney and only a small portion was deposited in bone. A third patient with Cushing's syndrome (adrenal cortical adenoma) of 15 months' duration had moderate skeletal demineralization but no fractures. Her miscible pool was normal but the rate of urinary excretion was greatly increased and the bone deposition rate was a little above normal (Appendix Table VIII). After removal of the adrenal cortical adenoma, the urinary excretion rate was normal and bone deposition increased slightly.

In contrast to the osteoporosis of Cushing's disease and old age, hyperthyroidism and acromegaly both enlarged the exchangeable pool. In patients with hyperthyroidism the fraction of the pool turning over was also greater, but in the nine acromegalics it was the same as in the controls. Hyperthyroidism without hypercalcemia increased bone deposition without increasing urinary excretion. With hypercalcemia and normal renal function, bone deposition was increased and urinary excretion greatly augmented. In acromegalics the mineral went rapidly to bone and the urinary excretion rate was only slightly accelerated. No difference was found between the data of four

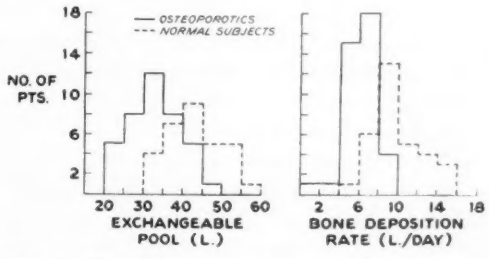

FIG. 3. FREQUENCY DISTRIBUTION CURVES FOR EXCHANGEABLE POOL SIZE AND BONE DEPOSITION RATES OF NORMAL SUBJECTS AND PATIENTS WITH POSTMENOPAUSAL OSTEOPOROSIS.

TABLE V

Effect of body size and weight on strontium dynamics

Condition	E	T	U	B
Body wt*				
Normals, average activity	0.602 ± 0023	0.196 ± 0.010	0.055 ± 0.004	0.140 ± 0.007
Normal athletes	0.758 ± 0.029	0.278 ± 0.014	0.077 ± 0.004	0.206 ± 0.014
Osteoporotics	0.542 ± 0.016	0.149 ± 0.007	0.041 ± 0.003	0.108 ± 0.006
Body size†				
Normals, average activity	23.2 ± 0.7	7.5 ± 0.3	2.1 ± 0.2	5.4 ± 0.2
Normal athletes	29.8 ± 1.2	10.9 ± 0.6	3.0 ± 0.1	7.9 ± 0.5
Osteoporotics	20.1 ± 0.6	5.6 ± 0.3	1.5 ± 0.1	4.0 ± 0.2

* Measurements of T, U and B are given as L/kg body weight/24 hrs; E is in L/kg.
† Measurements of T, U and B are given as L/m² BSA/24 hrs; E is in L/m².

acromegalics with roentgenographic evidence of osteoporosis and five without. In patients with Paget's disease of bone, the exchangeable pool and bone deposition rate were increased. The massive calcium deposits in one patient with chronic vitamin D overdosage increased the exchangeable pool almost tenfold; serum strontium values declined too rapidly to permit accurate measurement of bone deposition rate.

The reproducibility of the method is shown in Figure 4 where the results of a first test are plotted against a second test 21 times. For most subjects the values cluster fairly close to the theoretical slope of unity with a mean variation of 8 per cent.

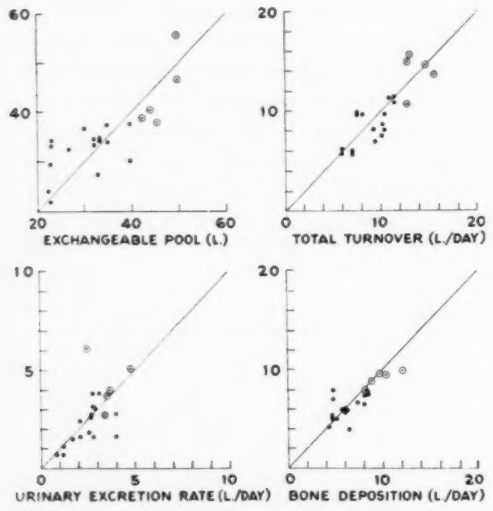

FIG. 4. REPRODUCIBILITY OF SKELETAL DYNAMICS MEASURED BY STABLE STRONTIUM. Duplicate (and occasionally triplicate) tests were performed 16 times in osteoporotic patients (dots) and in 5 normal subjects (circled dots). First results are plotted on abscissa and second (and third) tests on ordinate.

DISCUSSION

The data indicate that strontium can be used as a tracer to detect alterations in skeletal metabolism. In order to relate changes in bone deposition of strontium to those of calcium, any major difference in their metabolism must be accounted for. Major differences do occur in absorption and excretion and there are minor differences in distribution. The intestine discriminates greatly against *orally* administered strontium (30) but fecal losses of intravenously injected Ca^{45} and Sr^{85} are not widely divergent (11, 30). Renal clearance of strontium is three to five times that of calcium (12) and renal discrimination appears responsible for the difference in total retention of the two elements when they are given intravenously. Skeletal accumulation of radioactive Ca and Sr was equal in rabbits whose kidneys had been poisoned with mercuric chloride (31) and in nephrectomized rats (32). The distribution of strontium is quite similar to that of calcium. Double tracer experiments in man with Ca^{45} and Sr^{85} gave similar "pool" sizes and disappearance rate constants for the two elements (11). Most of an injected dose of either element is deposited in bone (23, 26, 27, 33), and radioactive isotopes of the two elements produce the same radioautographic patterns in bone (27, 34). Their passage from blood into extracellular fluid and spinal fluid are the same (24, 25), but intracellular penetration of strontium appears limited (Table I). Differences in protein binding of strontium and calcium have

been reported for man (35) but not for the rat (36).

Many studies suggest that bone discriminates but little between calcium and strontium *in vivo*. From a study in which Sr^{87} and Ca^{45} were given orally, it was inferred that in man the ratio of Sr^{87} to Ca^{45} was the same in bone and blood (37). Changes in Sr metabolism paralleled those for Ca in various diseases affecting skeletal metabolism (5, 11). When differences in urinary excretion were taken into account, Sr^{90} and Ca^{45} had no marked difference in bone deposition rates after their simultaneous administration to rats (38). Their skeletal accumulation is the same in anuric animals (31, 32) and in short-term experiments in intact animals (36). The two are also taken up to almost the same extent *in vitro* by surviving embryonic chick bone (39), bone powder (31) and synthetic apatite crystals (36). Equimolar exchange of strontium for the calcium of bone crystal has been shown (40). However, bone does not appear to handle the two elements identically in all respects. Preferential release of strontium from bone has been noted both *in vivo* (32, 36) and *in vitro* (36, 39, 41, 42). In the present method gastrointestinal discrimination was minimized by administering the strontium intravenously, and differences in renal excretion were accounted for by direct measurement of the urinary strontium excretion rate. Since the major differences in their metabolism are accounted for, the calculated bone deposition rates for strontium probably closely reflect those of calcium. Although the present method overestimates the deposition rates by approximately 12 per cent because fecal excretion is not subtracted, changes in skeletal metabolism in the conditions studied are similar to those found when radiocalcium was used as a tracer.

The 10 mEq dose of strontium used in these studies is larger than usual tracer doses but was the minimal quantity that produced serum levels that could be accurately measured by flame spectrophotometry for 4 to 6 days after injection. Fraser and his associates (15) studied the problem of whether such large doses act as a true tracer by repeating tests at several dosage levels in the same subjects. They found no effect of varying the amount of strontium. Harrison and co-workers (12) have shown that renal clearance of strontium is the same over a wide range of serum concentrations. The percentage of dose excreted in the feces appears to be in the same range when large doses of stable strontium and small doses of Sr^{85} (11, 12, 15, 30) are given.

The use of tracers to measure bone accretion rates or osteogenesis has been challenged because bone-seeking radioisotopes are concentrated in "hot spots," where new bone is being formed, and are deposited diffusely in dense, previously well mineralized bone (43–45). Hence, part of the tracer transferred from the pool to bone may be accounted for by new bone formation, and part by another process which has not been fully characterized. This process may represent a slow exchange, intracrystalline rearrangement, or diffuse accretion, and is included in the measurement of what is variously termed bone formation rate, accretion rate, and rate of osteogenesis. The extent to which this process affects the exponential disappearance of tracer from the rapidly exchangeable calcium pool is a matter of conjecture. While the diffuse label may not represent new bone growth, it does involve deposition of mineral ions which are important in maintaining the supporting function of the skeleton. For this reason, we have called the entire movement of tracer from the exchangeable pool to deeper layers of bone, the bone deposition rate.

Some authors have proposed correcting bone deposition rates for body weight. Since part of the exchangeable calcium is in extracellular fluid, it is possible that pool size is partly related to body size. To determine whether the smaller exchangeable pool of senile osteoporotics could result from their smaller size, we calculated the effect of correction for weight and surface area in these patients (Table V). The decreased pool size of osteoporosis is partly explained by the smaller size of the patients, but the difference in the rate of bone deposition between the controls and the osteoporotics was only slightly reduced by correcting the figures. Since weight and surface area of the athletes were the same as those of the control subjects at average activity, body size could not be responsible for the increased values seen in the athletes. Perhaps correction for lean body mass would be more appropriate. Fraser and his associates (15) expressed the volume of tracer distribution and rate of osteogenesis in "total body

plasma units." They calculated plasma volume as a constant fraction of body weight, so this unit is merely the volume of distribution corrected for body weight. Since plasma contains only part of the exchangeable pool and plasma volume is not known to affect calcium turnover, we have preferred to express the results in apparent liters of tracer distribution based on sampling the exchangeable pool via the serum.

It is difficult to correlate our results quantitatively with most reported studies of skeletal dynamics because of variation in experimental design, methods of calculation, and selection of patients. The study most comparable in experimental design is that of Fraser and co-workers (15) who used a similar model of calcium movement and nonradioactive strontium as a tracer. However, they employed a weight correction and expressed the results in "total plasma units." Their results may be recalculated to our terminology if the weight of each subject is known. Individual weights are given only for their normal subjects, who had exchangeable pools about 10 per cent lower and bone deposition rates approximately 30 per cent lower than ours. These discrepancies might be accounted for by the different method used to correct for loss of tracer during the mixing period and to integrate urine excretion rate. In making this correction for initial loss of tracer, Fraser and associates assumed that total body retention of strontium was linear on a semilogarithmic plot, but Norris, Tyler and Brues (46) have shown that total body retention of bone-seeking elements is a linear function only when plotted on log-log coordinates. Nordin (9), using a modification of Fraser's method, reported data on seven normal subjects. He included data from the first 24 hours in plotting the rate of fall of urine specific activity over a 7-day period. Plots of serum concentration of bone-seeking tracers do not indicate first order rate processes until after 24 hours. Inclusion of these values from the first 24 hours, failure to correct for urinary loss of tracer during mixing, and the method of calculating fecal loss in this period operate in different directions to various degrees and make his results difficult to interpret. Bauer, Carlsson and Lindquist (6), using a method of calculation analagous to ours, calculated exchangeable pool space and bone accretion rates in normal subjects from data provided by their own and other published studies. In only one group are the figures expressed in liters of serum. Four normal subjects in this group had exchangeable pools and bone accretion rates higher than our normal subjects but these four were in various stages of convalescence from fracture or nerve injury. The results from the other groups were calculated from specific activities and are expressed in grams of calcium. Since the serum calcium of each subject is not given, one can convert their figures to liters of serum only by assuming an average normal serum calcium of 5.0 mEq per L of serum. Since the high and low extremes of normal serum calcium in most laboratories are about 18 per cent apart, such an assumption would not lead to valid comparison. Conversely, if one assumes the ratio of Sr/Ca to be the same in all parts of the exchangeable pool and the rate of movement into bone to be the same for the two elements, one can convert the results of the present study to milliequivalents of calcium by multiplying the result for each subject by his serum calcium value. Such conversion gives our group of normal adult subjects slightly smaller exchangeable space and faster bone deposition rates than those recalculated by Bauer and associates (6) from the Ca^{45} data of Bronner, Harris, Maletskos and Benda (2) (1 patient), Krane, Brownell, Stanbury and Corrigan (3) (4 subjects) and the figures reported by Heaney and Whedon (7) for 1 normal adult. Some of these variances might result from the lower intracellular penetration of strontium (viz. Table I) and our overestimation of bone deposition by not measuring fecal excretion.

Because of the moderate variation in values for normal subjects reported by different authors it is apparent that any comparison of changes in abnormal states must be in reference to normal figures obtained by each group. Because of the expected biological variation within any group, the small number of normal subjects reported by most authors makes such comparison liable to reservation except in those abnormal states associated with dramatic alteration in skeletal metabolism, such as Paget's disease. In this condition only greatly increased exchangeable pool size and bone deposition rates have been reported (7, 8, 11). This is true by our method also, and is

in keeping with previous concepts of bone metabolism in this disease.

Hyperthyroidism is associated with negative balance of calcium and phosphorus, which in extreme cases may lead to roentgenographically evident osteoporosis and pathological fractures. It has recently been proposed that the osteoporosis of acromegaly and hyperthyroidism may be related to the unavailability of protein for the production of bone matrix and a resulting decrease in bone formation (47). Our data are not compatible with this proposal and confirm the enlarged pool of exchangeable calcium and rapid turnover rate found in hyperthyroidism by Krane and his associates (3), Heaney and Whedon (7), Dow and Stanbury (11), and Fraser and colleagues (15). Since bone is forming rapidly in these conditions, the development of osteoporosis must be due to even more rapid rates of osteolysis.

Osteoporosis is a common feature of hyperadrenocorticism. Elevation of the urinary excretion rate despite the reduced size of the pool in two patients with Cushing's disease suggests that bone dissolution is responsible for the demineralization. The patient with adrenocortical adenoma and symptoms of short duration had a very rapid urinary excretion rate and an apparent effort (unsuccessful) to compensate for increased bone dissolution by a slight increase in bone deposition rate. A similar rapid rate of urinary excretion and 15 per cent increase in bone deposition is an early feature of corticoid overdose in normal subjects (48).

Albright's hypothesis that bone formation is decreased in postmenopausal osteoporosis has recently been challenged (49–51). Patients with postmenopausal osteoporosis have been reported to have calcium (7, 9, 11) and strontium (11, 15) kinetics indistinguishable from those of normal subjects. Our data show that the rate of bone formation is decreased in osteoporosis and that this difference persists after correction for body size. The histograms for exchangeable pool size and bone deposition rate for the control subjects and osteoporotics show moderate overlap between the two groups, but the groups clearly comprise two populations. Our values for osteoporotics are similar to those reported by others. Hence, interpretation of these values as "low" depends on the relatively high values of our normal subjects compared with the published normal values discussed above. Lower than normal values for strontium retention by osteoporotics have been reported by others (6, 13, 16). Bone accretion rate of P^{32} is also decreased in osteoporosis (52). These data are in harmony with Albright's hypothesis that postmenopausal osteoporosis is due to decreased osteogenesis, but do not prove it, since the low rate of bone formation could reflect a decrease in skeletal mass, as in the two cases of advanced Cushing's disease. However, if the decreased skeletal mass of postmenopausal osteoporosis is due to an increased rate of bone resorption, calcium and strontium metabolism might resemble that of Cushing's disease, in which the urinary excretion rate is rapid in spite of skeletal depletion manifested by osteoporosis and decreased exchangeable pool size. In our experience, long-standing postmenopausal osteoporosis is rarely associated with increased calciuria. If postmenopausal osteoporosis is associated with increased bone resorption, as suggested by others, the urinary excretion rate might be normal if exogenous calcium was curtailed and bone deposition rate was normal. Hypercalciuria might result from normal bone resorption with decreased rate of bone deposition. This situation occurs in the osteoporosis of disuse or immobilization, which is regularly accompanied by marked hypercalciuria. Physical strain is considered to be a normal osteoblastic stimulus and its loss results in decreased osteogenesis. This concept is supported by the decreased bone accretion rate in an immobilized subject (6) and by the greatly increased bone deposition rates in our group of hard-playing athletes. The decreased bone deposition rate in postmenopausal osteoporosis, as in immobilization osteoporosis, probably reflects impaired osteogenesis, but it is not yet clear whether this is due to abnormal bone mineral metabolism or bone matrix formation.

Unlike patients with osteoporosis, all reported hyperparathyroid patients have had enlarged exchangeable calcium pools and rapid bone turnover rates (11, 15). This is not surprising, since there is more exchange surface in the actively metabolizing bone. Secondary increase in osteoblastic activity is thought to occur in hyperparathyroidism only when bone involvement is well developed and changes are roentgenographically

evident. In our four patients with hyperparathyroidism who had roentgenographic changes or rise in serum alkaline phosphatase or both, the exchangeable pools were large and bone deposition rates rapid. The 17 patients who had normal bones by X-ray and alkaline phosphatase tests but showed microscopic evidence of bone involvement had small but significant increases in strontium space and turnover. Seven patients with no microscopic foci of bone resorption had normal values for pool size and decreased rates of bone deposition. Thus, the degree of bone involvement in hyperparathyroidism measured by strontium movement correlates well with that found histologically. In 11 patients with recurrent nephrolithiasis who were distinguishable from patients with hyperparathyroidism only by their lack of hypercalcemia, the pool size and total turnover rate were normal, but more mineral was being channeled to urine than in normal subjects and bone formation was slightly decreased. Four patients studied by Fraser and co-workers (15) before and after cure of hyperparathyroid bone disease showed continued avidity for strontium for several months. One of our patients with a rapid initial bone deposition rate still had high values 1 week after operation. Four other patients had normal or low bone deposition rates before operation and these did not change after removal of parathyroid adenomas (Table IV). Although the number of liters of exchangeable pool being deposited daily in bone does not change, the fall in calcium concentration throughout the pool postoperatively results in less actual calcium being deposited in bone. The decrease in urinary excretion rate of strontium postoperatively parallels the decrease in hypercalciuria usually seen when hypercalcemia is corrected.

SUMMARY

1. Skeletal dynamics were calculated by usual dilution formulas, using stable strontium as a tracer, in 25 normal subjects, 14 athletes, 26 patients with postmenopausal osteoporosis, 28 with primary hyperparathyroidism, 3 with hyperadrenocorticism 8 with acromegaly, 7 with thyrotoxicosis, 11 with urolithiasis, 5 with Paget's disease of bone, and 1 with vitamin D poisoning. The technic requires that 10 mEq of strontium gluconate be injected intravenously and blood and urine concentrations be measured for 4 to 6 days.

2. In normal subjects the rapidly miscible pool was equivalent to 42.7 ± 1.1 L of serum, turning over at a rate of 13.5 ± 0.6 L daily, of which 3.9 ± 0.2 L was excreted by the kidney and 9.6 ± 0.4 L went to bone. Since only approximately 2.5 per cent of the pool is excreted in the feces daily, fecal excretion was not measured routinely. Good reproducibility was found in 21 duplicate studies.

3. Intense muscular exercise (athletes) was found to expand the pool greatly and to accelerate the rate of deposition in bone.

4. Kinetically, two divergent types of osteoporosis were differentiated. A small pool and low rate of bone deposition were found in postmenopausal osteoporosis and Cushing's disease of long duration. The large pool and rapid rate of bone deposition in thyrotoxicosis was confirmed and also found in acromegaly. In these two, excessive bone resorption is postulated. Urinary excretion rate was excessive in Cushing's disease, thyrotoxicosis, and acromegaly.

5. In hyperparathyroidism with clinically evident osteitis, expanded pools, greatly increased turnover, urinary excretion, and bone deposition rates were confirmed. In patients with normal roentgenographic appearance and phosphatase, bone involvement was shown by slight increase in bone deposition rate and microscopic foci of resorption on iliac crest biopsy. In seven patients without histological foci of resorption, the bone deposition rate was not increased.

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APPENDIX

Tables I through IX give clinical and experimental data on individual subjects. In each table % TRP = per cent renal tubular resorption of phosphate, k = serum strontium disappearance rate constant per day, E = exchangeable pool size in liters, T = exchangeable pool turnover in liters per day, U = urinary excretion rate in liters per day and B = bone deposition rate in liters per day.

APPENDIX TABLE I

Healthy subjects, average activity

Name	Sex	Age	Wt	Ht	Clinical notes	Serum Ca	Serum P	Serum Phosphatase	Urine Ca	% TRP	k	E	T	U	B
		yrs	*kg*	*cm*		*mEq/L*	*mg/100 ml*	*SJR units*	*mEq/24 hrs*						
JES	M	24	78.2	185	Normal San Quentin	5.25	3.7		7.4	78	0.319	51.2	16.3	3.9	12.4
SUL	F	25	79.9	175	Familial hirsutism; normal bones by X-ray	4.95	4.0	4.8	6.6	86	0.286	35.8	10.2	3.5	6.7
DYK	M	26	77.8	173	Normal San Quentin	5.15	2.7		8.0	81	0.415	38.3	15.9	4.3	11.6
FIL	M	27	76.4	170	Normal San Quentin	5.10	2.9		6.6	88	0.280	50.6	14.2	4.1	10.1
DEL	M	27	86.3	188	Normal	5.70	3.2	5.4	6.4	81	0.359	45.3	15.5	3.5	12.2
						5.85	3.6		7.5	82	0.255	37.8	13.6	3.7	9.9
STA	M	27	65.5	183	Normal San Quentin	5.35	4.2		7.7	79	0.336	51.4	17.3	5.4	11.9
WYA	F	29	51.6	155	Dermatitis, hands	5.05	3.4		8.7	76	0.262	32.9	8.6	3.1	5.5
EIS	M	29	65.9	146	Normal	5.35	3.2	4.9	7.9	81	0.330	43.8	14.5	4.8	9.7
		30	69.1			5.00	2.7		8.2	87	0.352	41.9	14.8	5.0	9.8
CAM	M	30	81.9	180	Normal San Quentin	5.30	4.1		6.9	85	0.304	45.0	13.7	4.0	9.7
HUT	M	33	81.9	183	Normal San Quentin	5.10	4.4		8.2	88	0.276	46.2	18.1	4.0	14.1
ZEK	M	33	68.3	178	Normal San Quentin	5.55	3.4		7.2	79	0.259	49.6	12.9	2.5	10.4
						5.25	4.0		8.2	83	0.335	46.4	15.6	6.1	9.5
BLA	M	34	80.5	183	Normal San Quentin	5.30	2.8		23.1	77	0.380	51.4	23.4	9.2	14.2
KIN	M	37	99.6	185	Normal San Quentin	5.00	3.5		4.4	83	0.238	40.8	9.7	3.0	6.7
STO	M	40	91.9	183	Normal San Quentin	4.90	4.2		4.7	93	0.289	42.2	10.6	2.4	8.2
						5.25	4.2		4.1	91	0.252	44.6	12.9	3.2	9.7
GOR	M	41	84.1	181	Normal	5.10	3.8	6.1	3.5	79	0.254	49.4	12.6	3.7	8.9
						5.25	4.1		8.9	87	0.232	55.4	12.9	4.0	8.9
TOR	F	42	53.6	171	Juvenile epiphysitis	5.00	3.5	5.6	8.2	88	0.285	43.4	12.4	4.1	8.3
REY	F	43	53.6	163	Contact dermatitis	5.10	3.5	3.4	8.8	78	0.347	31.0	10.7	3.1	7.6
ARB	F	48	53.2	163	Psoriasis	4.75	3.6	4.6	3.5	84	0.256	34.0	8.5	1.9	6.6
AND	F	49	65.0	165	Psoriasis	5.30	3.4		11.1	77	0.447	35.6	15.9	3.5	12.4
HOLC	F	52	64.1	170	Normal	5.10	3.9	3.8	9.1	84	0.336	40.9	13.7	3.5	10.2
CRO	F	53	62.0	161	Normal	5.10		4.7	11.9		0.333	50.4	16.6	4.2	12.4
SOO	F	61	75.0	153	Normal	5.20	3.3	5.0	6.8	90	0.281	39.0	11.0	2.7	8.3
MYE	F	71	68.8	151	Hypertension	5.80	2.7	2.0	12.7	79	0.385	36.0	13.8	5.5	8.3
HOLM	F	73	60.4	165	Osteoarthritis, mild	5.05	2.9	3.4	6.7		0.299	42.1	12.6	3.4	9.2
						5.30	3.0	3.8	5.9		0.274	38.7	10.6	2.7	7.9
TUC	F	80	50.9	160	Osteoarthritis, mild	5.00	3.6	6.2	7.4		0.318	35.0	11.1	3.4	7.7

APPENDIX TABLE II

San Quentin athletes *

Name	Sex	Age	Wt	Ht	Serum Ca	Serum P	Serum Phosphatase	Urine Ca	% TRP	k	E	T	U	B
		yrs	*kg*	*cm*	*mEq/L*	*mg/100 ml*	*SJR units†*	*mEq/24 hrs*						
STA	M	26	70.0	174	5.10	3.7		8.9	82	0.412	43.8	16.1	5.6	17.0
					5.40	2.9		7.0	75	0.367	54.8	22.6	4.6	11.5
REE	M	34	71.9	185	5.40	3.2		10.4	80	0.325	44.4	14.4	5.2	9.2
ROL	M	28	68.7	171	5.45	4.5		11.9	78	0.383	56.4	21.6	7.4	14.2
MAS	M	25	67.8	175	5.50	3.2		11.1	81	0.361	47.2	17.0	5.2	11.8
THO	M	28	74.2	175	5.40	4.0		13.7	85	0.347	50.0	17.4	5.8	11.6
WAT	M	25	81.0	180	5.35	3.9		11.7	83	0.425	58.6	24.9	7.3	17.6
YOU	M	33	73.7	173	5.05	3.3		11.1	81	0.398	61.6	24.6	5.8	18.8
DEL	M	28	69.6	170	5.15	4.2		11.3	87	0.425	49.5	21.1	5.2	15.9
NEA	M	25	73.4	182	5.20	3.4		11.2	82	0.286	71.8	28.6	4.9	23.7
BEL	M	30	79.4	177	5.15	2.5		10.2	76	0.318	68.2	21.7	5.7	16.0
LEE	M	31	67.9	170	5.15	2.9		12.6	75	0.368	60.2	22.2	6.5	15.7
BAR	M	30	91.8	177	5.45	4.6		11.9	83	0.265	64.3	17.1	4.9	12.2
					5.10	3.3		16.5	83	0.315	65.4	20.7	5.5	14.1

* All subjects were normal. † No data.

APPENDIX TABLE III

Multiple urinary tract stones

Name	Sex	Age	Wt	Ht	Clinical notes	Serum Ca	Serum P	Serum Phosphatase	Urine Ca	C_c/GFR	k	E	T	U	B
		yrs	*kg*	*cm*		*mEq/L*	*mg/100 ml*	*SJR units*	*mEq/24 hrs*						
BLI	M	43	80.0	169	Stones, 8 yrs	5.30	3.2	3.0	6.5	83	0.266	48.4	12.9	3.1	9.8
COR	M	46	73.9	162	Familial history of stones; stones, 14 yrs	5.45	2.8	6.0	17.0	81	0.382	29.5	11.3	5.9	5.4
GEN	M	39	71.9	179	Recurrent stones, 8 yrs; pyelonephritis	5.65	2.9	6.0	11.1	83	0.269	34.8	9.4	5.2	4.2
FRI	M	40	76.2	182	Immobilized 13 yrs before; asymptomatic renal calculi	5.45	2.9	5.8	7.8	72	0.316	43.1	13.6	4.6	9.0
HOP	M	55	80.0	171	Recurrent stones, 12 yrs; rheumatic heart disease	5.30	2.8	6.0	9.4	64	0.279	41.7	11.6	3.9	7.7
LEO	M	29	68.7	170	Recurrent stones, 2 yrs	5.50	2.6	3.0	9.0	77	0.382	34.3	13.1	4.5	8.6
PAL	M	58	76.0	170	Ca oxalate stones, 7 yrs	5.55	3.2	8.0	11.8	81	0.305	36.6	11.2	6.2	5.0
RAG	M	53	73.0	176	Stones, 14 yrs	5.30	2.4	7.0	11.1	60	0.360	40.4	14.5	6.2	8.3
SCH	M	32	69.0	173	Ca oxalate and phosphate stones; chronic pyelonephritis, 5 yrs	5.40	3.3	5.6	12.4	86	0.415	45.4	18.8	11.6	7.2
KNI	M	59	59.6	167	Bilat. stones, 6 yrs; arteriosclerosis	5.35	2.4	4.8	16.1	79	0.370	49.8	18.4	8.5	9.9
MOR	M	32	66.2	148	Renal tbc; stones, 9 yrs	5.00	3.0	10.0	4.5	70	0.396	28.6	11.3	2.8	8.5

APPENDIX TABLE IV

Postmenopausal osteoporosis

Name	Sex	Age	Wt	Ht	Clinical notes	Serum Ca	Serum P	Serum Phosphatase	Urine Ca	C_c/GFR	k	E	T	U	B
		yrs	*kg*	*cm*		*mEq/L*	*mg/100 ml*	*SJR units*	*mEq/24 hrs*						
MCD	F	57	57.7	155	Mult. compressions T and L spine; nontoxic nodular goiter	5.20	2.7	7.0	4.9	84	0.247	37.4	9.2	2.4	6.8
WAL	F	60	52.3	154	Guillain-Barre, 10 yrs; postmeno. fract. $T_{7,8,9,10}$ $L_{1,5}$	4.90	3.3	8.0	4.5	87	0.278	43.8	11.8	1.9	9.9
OLE	F	60	55.0	157	Fract. lumbar vertebrae and left hip	5.25	2.8		3.9	85	0.245	27.7	6.3	1.4	4.9
NEI	F	63	33.2	146	Multiple compressions; osteoporosis (biopsy); hysterectomy at 40	5.35	3.4	4.9	4.1	86	0.258	34.7	9.0	1.8	7.2
BER	F	65	69.5	155		5.10	3.0		6.9	84	0.319	35.6	11.4	2.7	8.7
BRI	F	67	76.4	156	Bilat. ovariectomy at 47; compression L_2	4.75	3.2	4.3	6.2	85	0.254	41.5	10.6	3.4	7.2
CAM	F	67	58.2	146	Generalized demineralization; Schmorl's nodes	5.25	3.2	6.0	1.8	84	0.245	29.0	7.1	0.9	6.2
JAC	F	67	70.0	160	Fract. $T_{10,12}$; urinary tract infection	5.20	4.0	6.0	3.9	95	0.295	34.8	10.3	2.1	8.2
						5.10			3.0		0.237	33.8	8.0	1.6	6.4
			69.6			5.10			4.6		0.261	37.2	9.7	2.4	7.3
KRU	F	68	48.6	153	Demineralized spine; fract. $T_{8,10}L_1$; thyrotoxic, 25 yrs previous	5.25	3.0		7.0	88	0.347	28.8	10.0	3.3	6.7
GIB	F	68	72.8	159	Femoral fract.; diabetes	5.05	3.5	3.3	2.5	91	0.181	40.4	7.3	2.0	5.3
AND	F	69	54.5	163	Osteoporosis (biopsy); demineralized spine; emphysema	5.40	4.3	7.2	5.0	95	0.199	35.1	7.0	5.4	1.6
BRU	F	70	54.1	145	Comp. fract. T_6-L_2; calcification in spleen and liver	5.15	4.2	5.2	6.6	82	0.316	32.1	10.1	4.0	6.1
						4.95	3.5		4.9		0.259	33.2	8.6	2.8	5.8
			53.9			5.05	3.2		4.1	87	0.219	34.4	7.5	1.6	5.9

APPENDIX TABLE IV—(*Continued*)

Postmenopausal osteoporosis

Name	Sex	Age	Wt	Ht	Clinical notes	Serum Ca	Serum P	Serum Phosphatase	Urine Ca	% TRP	*k*	*E*	*T*	*U*	*B*
		yrs	*kg*	*cm*		*mEq/L*	*mg/100 ml*	*SJR units*	*mEq/24 hrs*						
DAD	F	70	52.3	158	Mod. demineralization; biconcave vertebrae	5.10	4.0	5.7	9.8	88	0.300	39.7	11.3	2.8	8.5
			53.4			5.00	3.4		7.0	91	0.359	30.2	10.8	3.1	7.7
			53.0			4.85			7.8		0.286	37.5	11.3	3.8	7.5
MEY	F	70	66.3	155		5.20			3.2		0.308	30.0	9.2	1.7	7.5
			66.5			5.10			2.6		0.223	36.5	8.1	1.5	6.6
VIC	F	72	67.3	157	Osteoarthritis; Schmorl's nodes; arteriosclerosis	5.35	3.5	5.0	6.1	79	0.286	32.8	9.4	2.9	6.5
			68.5			5.45			6.4		0.318	27.3	6.9	3.0	3.9
WAG	F	72	74.0	155	Compressions L_{1-4}; Parkinson's dis.; arterioscler.	5.05	3.7	7.6	8.1	86	0.267	36.2	9.7	5.0	4.7
COO	F	74	50.0	154	Osteoporosis (biopsy); treated lues	5.30	3.4	4.3	2.2	87	0.262	26.6	7.0	0.8	6.2
						5.25	2.7		1.7	80	0.172	32.3	5.6	0.7	4.9
WAT	F	75	38.1	144	Osteoporosis (biopsy); gastrectomy	4.55	3.4	6.0	0.8	90	0.187	45.2	8.5	0.8	7.7
MAN	F	75	53.2	165		5.15			4.1		0.208	34.7	7.2	1.5	5.7
WALK	F	76	46.8	158		5.10	3.4		3.8		0.248	28.8	7.2	1.4	4.8
STA	F	77	59.5	146	Collapsed $T_{10,12}$ $L_{2,4}$; scoliosis; osteoarthritis; arteriosclerosis	5.25	3.7		1.6		0.260	22.8	5.9	1.2	4.7
			60.4			5.10			1.7		0.282	22.0	6.2	1.1	5.1
		78	60.9			5.05			4.1		0.193	29.3	5.7	0.7	5.0
MCC	F	78	59.5	147	Thyrotoxic, 13 yrs prev.; compression T and L spine	4.80	3.6		4.1	89	0.328	21.3	7.0	2.6	4.4
			59.0			4.70	3.2		3.3	81	0.245	24.1	5.9	1.8	4.1
CHA	F	80	59.1	158	Compression $T_{12}L_{1,2}$	5.25	3.5		5.5	83	0.327	23.0	7.5	2.7	4.8
			60.3	157		5.20	3.0		5.7		0.290	33.2	9.6	2.6	7.0
			60.2			5.20			3.9		0.284	34.1	9.7	2.7	7.0
JAE	F	80	62.1	155	Compressed L vertebrae; emphysema	4.70	2.7	8.0	4.3	84	0.258	41.2	16.0	1.8	14.2
SCH	F	81	61.2	167	Anterior compression L_{2-4}, bromide rash	4.90	2.8	6.8	1.5	79	0.270	30.2	8.2	3.1	5.1
BRUS	F	85	56.6	151	Schmorl's nodes	5.35	3.4	4.0	3.4	82	0.215	40.7	8.8	2.2	6.6

APPENDIX TABLE V

Hyperthyroids

Name	Sex	Age	Wt	Ht	Clinical notes	Serum Ca	Serum P	Serum Phosphatase	Urine Ca	% TRP	*k*	*E*	*T*	*U*	*B*
		yrs	*kg*	*cm*		*mEq/L*	*mg/100 ml*	*SJR units*	*mEq/24 hrs*						
CLE	F	33	50.0	168	Graves' disease	5.25	4.8	7.0	3.6	82	0.468	52.0	24.3	4.4	19.9
SYK	M	70	56.7	160	Toxic nodule	5.35			4.3		0.392	51.4	20.2	2.4	17.8
PAP	F	59	54.8	153	Menopause at 33; osteoporosis; toxic nodular goiter; comp. of T_{11-12}	5.20	3.3	3.0	2.4	82	0.292	41.1	12.0	1.5	10.5
SMI	F	46	60.7	167	Constipated; thirsty; Graves' disease	5.75	4.4	6.7	19.4	91	0.488	55.9	27.2	4.6	22.6
GIL	M	32	54.5	170	Graves' disease with myopathy; osteitis (biopsy)	6.22	4.2		40.7	76	0.693	48.7	33.7	16.2	17.5
SVA	F	53	42.2	160	Graves' disease; fract. T_9, L_1	5.75	5.0	6.0	16.9	88	0.592	61.2	36.2	14.0	21.8
MAR	F	58	57.1	160	Graves' dis.; uremia; comp. fract. $T_{7,11,12}$	6.71	3.7	4.7	20.0		0.401	32.2	12.9	6.0	6.9

APPENDIX TABLE VI

Hyperparathyroidism

Name	Sex	Age	Wt	Ht	Clinical notes	Serum Ca	Serum P	Serum Phosphatase	Urine Ca	% TRP	*k*	*E*	*T*	*U*	*B*
		yrs	*kg*	*cm*		*mEq/L*	*mg/100 ml*	*SJR units*	*mEq/24 hrs*						
Hyperparathyroidism with high alkaline phosphatase level or osteitis by X-ray															
ABR	F	69	57.5	148	Stones-bilat.; Paget's disease of bone	6.05	2.8	15.0	10.7	72	0.269	68.2	18.4	4.7	13.7
OLD	M	50	76.3	174	Giant cell osteoclastoma; partial loss of lamina dura	7.15	1.1	11.0	19.6	58	0.413	50.8	21.0	8.2	12.8
DEL	M	29	60.9	161	Demineralized skeleton; no osteitis (biopsy); stones rt. side	7.00	2.2	11.9	16.4	75	0.517	65.0	33.6	8.8	24.8
LEV	M	40	70.5	171	Stones, left. Duodenal ulcer	6.90	2.4	10.1	24.1	70	0.430	73.7	31.7	7.3	24.4
Hyperparathyroidism with normal X-ray appearance, normal alkaline phosphatase level, but positive bone biopsy															
BRO	M	52	78.8	180	Stones, bilateral	6.20	3.1	3.8	26.8	67	0.217	79.1	17.2	8.1	9.1
HOE	F	46	61.2	157	Stones, bilateral	6.75	1.7	7.7	13.1	70	0.357	40.7	14.5	4.8	9.7
JON	M	38	80.0	173	Kidney stones	6.05	2.3	5.2	21.6	75	0.359	61.5	22.0	8.3	13.7
MOO	F	50	75.0	165	Parathyroid cyst palpated in neck; no stones	6.35	3.0	5.0	18.8	71	0.375	54.6	20.6	8.4	12.2
NOR	M	40	61.4	178	Stones, bilateral	7.00	2.4	6.2	31.7	75	0.359	49.9	17.9	6.3	11.6
POL	M	20	69.2	182	Stones, bilateral	7.35	2.4	8.4	25.4	69	0.502	58.2	29.2	10.0	19.2
			69.0			7.05	2.4	6.0	25.0	70	0.405	57.4	23.2	7.0	16.2
PUR	F	54	62.5	167	Stones, bilateral	6.60	2.5	7.1	19.7	66	0.333	51.6	17.2	4.5	12.7
DAR	F	53	69.5	160	Renal stones, left	5.85	3.2	2.5	8.7	65	0.403	33.5	13.5	4.2	9.3
ALV	F	60	54.9	160	Constipation, nausea	6.25	3.3	5.6	11.4	72	0.335	48.5	14.5	5.0	9.5
NOR	M	56	68.4	164	Stones, bilateral	7.35	2.2	6.5	21.9	70	0.509	32.6	16.5	6.2	10.3
CAN	F	37	59.4	163	Inc. fatigue, 14 mos Stones, left, 18 mos	6.00	2.8		9.1	81	0.367	54.2	16.6	3.4	13.2
HYA	F	35	40.4	151	Stones, bilateral	7.20	2.9	6.2	25.3	75	0.722	24.6	17.8	8.0	9.8
NEL	M	34	71.2	171	Stones, bilateral	6.10	2.5	3.7	18.7	71	0.431	45.5	19.6	9.3	10.3
COR	M	55	85.6	177	Stones, bilat. ulcer hist.	6.30	2.3	6.1	10.1	82	0.415	43.2	17.9	10.0	7.9
WOL	F	52	56.0	160	Stones, bilat.; prev. hemorrhage into parathyroid cyst; ulcer	6.65	2.5	6.9	4.3		0.308	51.2	15.8	4.4	11.4
BRY	F	58	120.2	163	Weakness, nausea, emesis, no stones	6.05	2.2	4.3	17.6	62	0.324	55.2	17.9	8.7	9.2
HOD	F	32	57.7	169	Nephrolithiasis; nephrocalcinosis, bilat.	6.20	3.0	6.3	23.2	67	0.403	50.0	20.2	6.4	11.8
Hyperparathyroidism with normal X-ray appearance, normal alkaline phosphatase level, and normal bone biopsy															
ALE	F	56	59.1	148	Back pain; gen. weakness and body aches, 5 yrs	6.25	1.4	5.7	8.0	70	0.350	28.7	10.0	2.9	7.1
DYM	F	56	86.5	162	Stones, bilateral	5.85	2.0	6.5	11.6	64	0.238	53.1	12.6	3.5	9.1
LAM	F	51	74.5	163	Stones, bilateral, 2 yrs	6.10	2.6	2.0	14.6	76	0.359	31.7	11.4	5.5	5.9
SWE	F	70	50.7	152	Radical mastectomy for cancer; constipation; fractured vertebrae with demineralization; osteoporosis (biopsy)	7.05	2.3	6.8	11.0	53	0.245	35.8	8.8	2.7	6.1
TUR	M	25	91.5	175	Back pain, juvenile epiphysitis	6.15	2.2	6.0	23.2	69	0.389	43.4	16.9	5.8	11.1
SAU	M	43	67.1	170	Stones, bilat.; serum Ca fell to normal on corticoids	6.10	1.9	5.9	12.3	71	0.308	45.2	13.9	5.2	8.7
MCB	M	50	75.6	169	Uremia; renal stones	6.55	2.5	6.5	12.1	74	0.276	33.5	9.2	4.6	4.6

APPENDIX TABLE VII

Acromegaly *

Name	Sex	Age	Wt	Ht	Serum Ca	Serum P	Serum Phosphatase	Urine Ca	% TRP	k	E	T	U	B
		yrs	*kg*	*cm*	*mEq/L*	*mg/100 ml*	*SJR units*	*mEq/24 hrs*						
ODL	F	35	8.55	165	5.20	5.0		6.4	95	0.308	64.7	18.5	5.4	13.1
		36			5.35	4.8		9.4		0.286	72.4	19.2	7.5	11.7
BOL	F	30	56.3	155	5.20	4.7	5.5	7.9	94	0.425	39.7	16.9	5.8	11.1
WHI	F	38	72.7	163	5.25	4.1		10.6	82	0.322	66.2	21.3	5.4	15.9
LOH	M	29	100.0	203	5.20	4.4	6.6	13.8	89	0.425	103.5	41.0	7.7	33.3
SEG	M	47	85.8	118	5.35	4.6		11.9	83	0.401	73.2	29.3	5.2	24.1
THO	M	41	78.5	174	5.15	3.4		7.3		0.289	77.2	22.3	5.8	16.5
BAL	M	44	72.7	165	5.15	5.2		6.5	91	0.245	70.8	17.4	2.5	14.9
REE	M	55	78.7	171	5.30	3.8	6.0	15.8	80	0.284	96.8	27.5	6.0	21.5

* Clinically active in all patients.

APPENDIX TABLE VIII

Adrenocortical hyperfunction

Name	Sex	Age	Wt	Ht	Clinical notes	Serum Ca	Serum P	Serum Phosphatase	Urine Ca	% TRP	k	E	T	U	B
		yrs	*kg*	*cm*		*mEq/L*	*mg/100 ml*	*SJR units*	*mEq/24 hrs*						
ARM	M	57	59.3	167	Bilateral hyperplasia	5.00	2.8	4.1	6.0	78	0.537	28.2	15.2	8.6	6.6
CHU	F	57	50.5	174	Bilateral hyperplasia	5.30	3.4	6.8	5.9	84	0.413	19.8	8.2	5.8	2.4
GIL	F	26	73.3	162	Left adrenal adenoma; diabetes; obesity; amenorrhea; demineralized spine, fract. T_8	5.35	2.7	3.4	23.3	80	0.846	41.1	34.8	20.2	14.6
	3 mos post op.		67.1	160	No remineralization; collapse T_{12}; on cortisol, 0.015 daily	5.25			2.9		0.430	46.1	19.8	2.4	17.4

APPENDIX TABLE IX

Paget's disease

Name	Sex	Age	Wt	Ht	Clinical notes	Serum Ca	Serum P	Serum Phosphatase	Urine Ca	% TRP	k	E	T	U	B
		yrs	*kg*	*cm*		*mEq/L*	*mg/100 ml*	*SJR units*	*mEq/24 hrs*						
SAN	M	54	56.5	164	Prog. muscular dystrophy, familial; pelvis, femora, calcanei	5.35	3.8	18.9	6.2	86	0.423	69.8	29.5	3.3	26.2
MAR	M	41	65.7	164	Marked widespread disease	5.05	3.6	178.0	3.6	87	1.04	175.5	182.5	6.8	175.7
SIM	M	46	81.5	118	L_3, rt. femur	5.00	4.1	3.0	7.4		0.336	65.0	21.8	5.5	16.3
MAC	M	74	81.0	181	L clavicle; rt. scapula; L tibia; skull; pelvis	5.45	2.9	32.5	7.8		0.347	125.0	43.4	5.8	37.6
MCK	M	76	44.0	172	Pelvis and lumbar spine	5.00	3.1	9.0	4.0	85	0.396	41.5	16.4	2.3	14.1

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A HUMAN HEMOGLOBIN WITH LOWERED OXYGEN AFFINITY AND IMPAIRED HEME–HEME INTERACTIONS *

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The reactivity of the iron in the four hemes of the mammalian hemoglobin molecule depends largely on the interactions between the prosthetic groups and the amino acid chains of the globin (1, 2). Normal configuration of heme groups in the abnormal human hemoglobins, therefore, does not preclude anomalies of their oxygen affinity which might arise from aberrations in the globin. In most of the more than 20 recognized abnormal human hemoglobins the amino acid abnormality does not affect the oxygen affinity. Amino acid changes which are remote from groups crucial in the globin-heme interaction do not affect oxygenation. Abnormal oxygen affinities have been reported in hemoglobin H and M. Hemoglobin H contains only the beta and not the alpha chains of normal hemoglobin and exhibits an oxygen affinity ten times greater than normal but has no Bohr effect (3). Hemoglobin M causes cyanosis apparently due to its inability to combine with oxygen. The pigment may be present in the blood as methemoglobin M or as its nonoxidized precursor (4).

We have recently encountered a hemoglobin which caused cyanosis due to an abnormally low oxygen affinity. The entire oxyhemoglobin dissociation curve was markedly shifted, but at sufficiently high oxygen tensions the pigment became fully saturated. The oxygen equilibria, spectroscopic and preliminary electrophoretic findings are presented. Since a lowered oxygen affinity of hemoglobin is usually not considered in the differential diagnosis of cyanosis, some reference is made to the methods which lead to its detection.

PROPOSITUS

The patient, a 14 year old white male of normal growth and superior intelligence, has been cyanotic since birth. No clubbing, squatting or shortness of breath has ever occurred, but he has had some episodes of weakness after severe exertion. Pulmonary function tests, including diffusion studies, were within normal limits. Hemoglobin was 13.9 g per cent; erythrocytes 4.6 million; reticulocytes 1.4 per cent; red cell morphology normal. Examination of the blood for methemoglobin or sulfhemoglobin was negative. Cardiac catherization, including dye injection studies, revealed on three different occasions low arterial and venous oxygen content but no evidence of cardiovascular abnormalities.

The patient is an only child. His grandparents are deceased; his father's hemoglobin is normal. His mother's hemoglobin showed almost quantitatively the same abnormalities as that of the patient. One brother and sister of the mother as well as one male and one female descendent of the brother have normal hemoglobin A.

METHODS

Arterial and venous blood samples were taken during inhalation of various O_2–N_2 mixtures. All gas samples were analyzed in an E_2 Beckman oxygen analyzer. The oxygen tension of blood was determined with a Clark-Severinghaus oxygen electrode (5) (Yellow Springs Instrument Corp.) and the oxygen and carbon dioxide of the blood by the manometric technique of Van Slyke and Neill. Blood pH was measured at 37° C in an anaerobic electrode with a Beckman model M pH meter. The determination of the oxygen capacity of the blood under these circumstances required special attention to the following possibilities: *1)* the usual equilibration of blood *in vitro* may not fully saturate the heme pigments in the sample and *2)* the spectrophotometric measurements of the hemoglobin concentration may not be valid when dealing with an abnormal pigment with unknown light absorption constants. The total hemoglobin concentration was therefore ascertained by three methods: *1)* spectrophotometric hemoglobin iron determination after wet ashing (6) and calculation of the pigment concentration on the basis of hemoglobin iron concentration of 0.335 per cent; *2)* gasometric measurement of the oxygen capacity after equilibration of the blood *in vitro* with oxygen at 20° C (1.34 cc oxygen equivalent to 1 g hemoglobin); and *3)* spectrophotometric measurement of the pigment after its conversion into cyanmethemoglobin, applying a millimolar extinction coefficient of 11.5 at 542 mμ (7). Absorption spectra of the undiluted blood after anaerobic lysis with saponin were obtained using a cuvet of 0.0087 cm thickness (Waters-Conley Corp.) in a Beck-

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man DU spectrophotometer. Ultraviolet spectra were obtained with a Cary recording spectrophotometer. All optical densities were expressed per 1 cm light path and as the concentration of 1 mmole per L of the subunit (mol wt 16,700) of hemoglobin. One mmole per L of the subunit corresponds to 1.67 g per cent hemoglobin (mol wt 67,000).

Hemoglobin solutions were prepared at 4° C. After repeated washings with saline, the cells were lysed with approximately twice their volume of distilled water plus 5 per cent toluene. After centrifugation at 25,000 G for 30 minutes, the decanted clear hemoglobin solution was mixed with buffer of desired pH and ionic strength. Oxygen equilibria studies were carried out on freshly prepared hemoglobin solutions of approximately 6 g per cent concentration in 0.15 M phosphate buffer. Samples were equilibrated in a tonometer with O_2–N_2 mixtures at 37° and 28° C water-bath temperatures for 20 minutes. The hemoglobin solution was then transferred anaerobically into the 0.0087 cm cuvet, and oxyhemoglobin (HbO_2) and reduced hemoglobin (Hb) fractions were determined spectrophotometrically according to the method of Drabkin (8). The absence of methemoglobin was verified in each sample by preparation of a companion sample with addition of freshly neutralized KCN and comparison of the two absorption spectra.

Spectrophotometric studies on methemoglobin (MetHb) were made on dilute solutions in phosphate or borate buffer after oxidation of the pigment at pH 6.5 by potassium ferricyanide which was added to the hemoglobin solution in molar ratio of 6 to 1. The percentage of alkaline and acid methemoglobin at a given pH was ascertained according to the method of Austin and Drabkin (7). All pH values were corrected to 0.1 ionic strength. The methemoglobin dissociation was calculated according to the equation $pH = pK + \log$ (alkaline MetHb/acid MetHb).

Electrophoretic studies were done on granular or gel starch blocks, cooled with circulating ice water (9). A current of approximately 40 ma at 250 v was applied. Electrophoresis of oxyhemoglobin, carboxyhemoglobin, methemoglobin and cyanmethemoglobin was studied at pH 8.6 (veronal buffer, 0.05 ionic strength) and at 7.4, 7.05 and 6.5 (phosphate buffer, 0.05 ionic strength).

RESULTS

In vivo oxygen saturation of the blood. While the patient was breathing room air his arterial oxygen tension was normal and ranged between 96 and 104 mm Hg. The oxygen content of the arterial blood, after subtraction of the oxygen in physical solution, increased by about 35 per cent between room air and 100 per cent oxygen breathing, clearly indicating incomplete saturation of the hemoglobin at a usual arterial oxygen tension of 100 mm Hg. After equilibration of the blood *in vitro* at 20° C with oxygen, its gasometrically measured oxygen capacity agreed satisfactorily with its capacity calculated on the basis of the heme iron determination (Table I) indicating that all hemes present had combined with oxygen. An *in vivo* oxygen saturation curve (Figure 1) was constructed accordingly and demonstrated an enormous shift to the right. There was saturation of 60 per cent in the arterial blood at a normal P_{O_2} of 100 mm Hg, and a saturation of 94 per cent during oxygen inhalation. An *in vitro* dissociation curve of the blood showed essentially the same results.

TABLE I

Total hemoglobin measured as hemoglobin iron, cyanmethemoglobin and oxygen capacity*

	Gasometric† (vol % O_2/1.34)	Hemoglobin-iron (mg % Fe/3.35 $\times 10^{-2}$)	Cyanmethemoglobin (mmoles/L =11.5 at 542 mμ)
Patient	13.91 ± 0.14	14.02 ± 0.09	13.98 ± 0.09
Normal	14.24 ± 0.16	14.30 ± 0.12	14.32 ± 0.08

* Figures given are given in g % as mean ± standard deviation of three determinations.

† After equilibration of the blood with 100% O_2 at 20° C.

While the patient was breathing room air the pH of the arterial blood was 7.38 and its CO_2 content 22.2 mmoles per L. The CO_2 content of the arterial plasma was 27.3 mmoles per L, indicating a normal distribution of the CO_2 between plasma and corpuscles.

Oxygen equilibria of hemoglobin preparations in buffer solutions. Hemoglobin, freed of stromata and lipids, in 0.15 M phosphate buffer solution was equilibrated against known O_2–N_2 mixtures

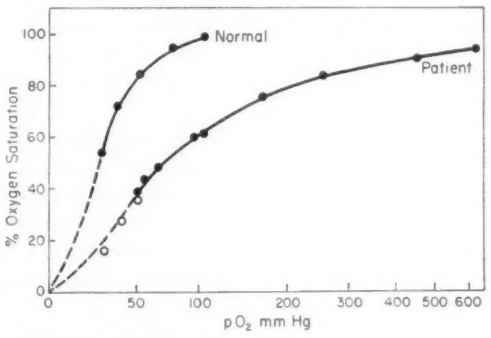

FIG. 1. *IN VIVO* OXYGEN SATURATION OF ARTERIAL BLOOD DURING INHALATION OF O_2–N_2 MIXTURES; BLOOD PH 7.34 TO 7.39. Open circles are measurements in venous blood.

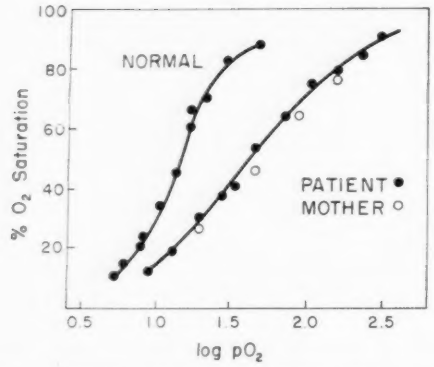

FIG. 2. OXYGEN EQUILIBRIA OF HEMOGLOBIN (APPROXIMATELY 6 G PER CENT) IN 0.15 M PHOSPHATE BUFFER AT 37° C. pH: patient, 7.37; mother, 7.36; normal control, 7.39.

and the resultant dissociation curves are presented in Figure 2. As observed by others (10, 11), the oxygen affinity of freed hemoglobin was considerably greater than that of intact red cells, a phenomenon probably due to differences in pH, CO_2 and ionic concentrations in the environment of the hemoglobin molecules. As a dilute hemoglobin solution, normal hemoglobin A required 14 mm Hg P_{O_2} to produce 50 per cent saturation versus 40 mm Hg for the patient's hemoglobin. As intact blood, 50 per cent saturation points were at P_{O_2} of 25 mm Hg for the normal control compared with 70 mm Hg for the patient. In other words the patient's hemoglobin in buffer solutions as well as in intact cells required oxygen tensions roughly 2.8 times greater than normal for half-saturation. Four-hour dialysis of the hemoglobin solutions prior to the equilibration did not materially alter the oxygen equilibria. Hemoglobin solutions prepared from blood of the patient's mother showed a dissociation curve almost identical with that of the patient.

The grossly abnormal shape of the patient's dissociation curve in Figure 2 should be noted. The asymmetry of the curve was not caused by the presence of methemoglobin or by partial denaturation of the hemoglobin. Both conditions, which are common causes of asymmetrical curves, were eliminated by spectroscopic examinations.

The steepness of the normal curve in the plot of oxygen saturation versus log P_{O_2} (Figure 2) is related to heme-heme interactions. A numerical expression of the intensity of these interactions can be obtained from Hill's equation: $\log (y/100 - y) = \log K + n \log P_{O_2}$ where y is the per cent oxyhemoglobin, n is an expression of the heme interactions ($n = 1$ = no interactions), and K is a measure of the affinity for oxygen. The n value was calculated for the slope in the range of 50 per cent saturation and was 2.6 for normal hemoglobin and 1.1 for the patient's hemoglobin.

The effect of pH on the oxygen equilibria of the patient's hemoglobin was not different from that on the normal control (Figure 3). These measurements were made at 28° C and a comparison with measurements at 37° C indicates a somewhat smaller temperature effect on the affinity of the abnormal hemoglobin than on the normal.

Spectroscopy of the native pigments. Spectrophotometric examinations were made with the following objectives: *1*) to ascertain the nature of the pigment which remained unoxygenated in the patient's blood *in vivo* and *2*) to obtain ultraviolet absorption spectra with particular attention to signs of possible denaturation of the pigment.

After complete saturation with oxygen *in vitro*, the blood was quickly hemolyzed and examined in the 0.0087 cm cuvet. Its absorption spectrum was identical with that of normal oxyhemoglobin (Figure 4). After evacuation at zero oxygen tension, a normal reduced hemoglobin absorption spectrum was found. Arterial and venous blood

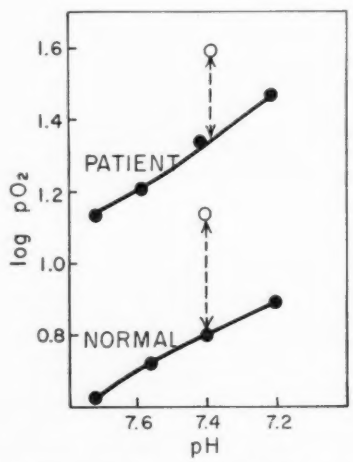

FIG. 3. EFFECT OF PH AND TEMPERATURE ON HALF-SATURATION ($HbO_2 = Hb$) OF HEMOGLOBIN (APPROXIMATELY 6 G PER CENT) IN 0.15 M PHOSPHATE BUFFER. Solid circles at 28° C, open circles at 37° C.

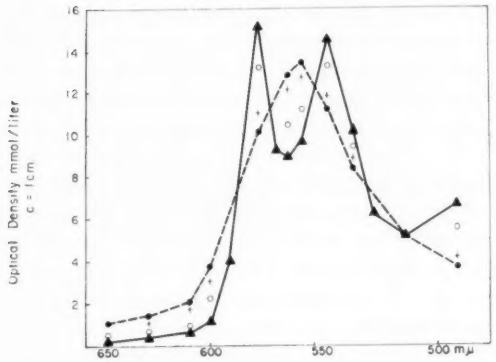

FIG. 4. ABSORPTION SPECTRA OF THE PATIENT'S HEMOLYZED BLOOD (UNDILUTED IN 0.0087 CM CUVET). ▲—▲ after equilibration with 100% O_2 at 20° C; ●--● after equilibration at zero P_{O_2}; + + native venous blood; ○ ○ native arterial blood.

samples of the patient were examined under anaerobic conditions on six different occasions and failed to reveal the presence of any pigment other than oxyhemoglobin and hemoglobin. Two examples are indicated in Figure 4. The light absorption of arterial blood closely fitted a theoretical curve representing 59 per cent oxyhemoglobin and 41 per cent hemoglobin. Venous blood followed a predicted curve of 18 per cent oxyhemoglobin and 82 per cent hemoglobin. Although these absorption spectra were obtained within 10 minutes after shedding of the blood, the remote possibility remained that a circulating methemoglobin might have been reduced during this time, perhaps due to a delayed reductase activity. Blood was therefore collected with and without KCN present in the sampling syringe. No spectral differences referable to the formation of cyanmethemoglobin were noted.

The absorption spectra in the ultraviolet spectral region, presented in Figure 5, were obtained from the hemoglobin preparations used in the study of the oxygen equilibria. They failed to show any indication of denaturation. The peak of the Soret band of oxyhemoglobin was at 415 mμ and that of the hemoglobin at 425 mμ. The tryptophan and phenylalanine bands were at the same wave lengths and of similar amplitude as the bands observed in the normal controls.

Absorption spectra of the ferri-hemoglobins. The methemoglobin produced by *in vitro* oxidation of the hemolysates from the patient and his mother exhibited identical abnormalities (Figure 6). The greater optical density of the patient's methemoglobin (pH 6.5) in the green spectral region is not caused by incomplete oxidation of some hemes, because it extends into the 530 to

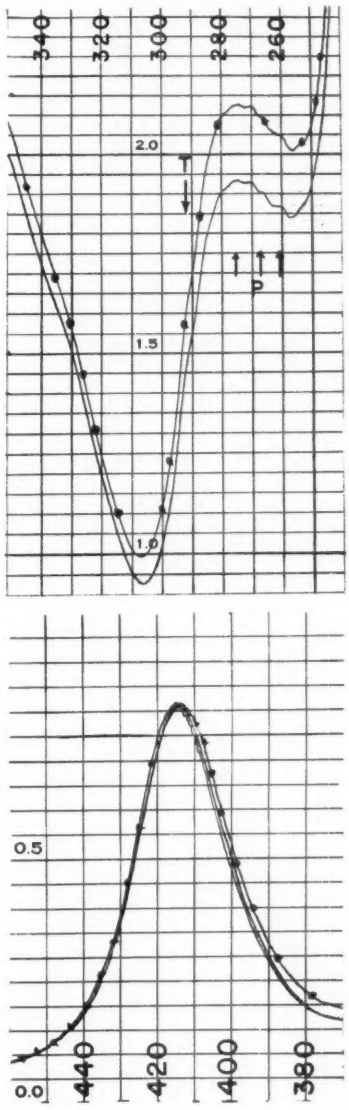

FIG. 5. ULTRAVIOLET ABSORPTION SPECTRA OF HEMOGLOBIN SOLUTIONS. The curve marked by solid circles is that of the patient. Lower: Soret band of HbO_2; upper: tryptophan (T) and phenylalanine (P) bands of Hb. (Wave lengths in millimicrons.)

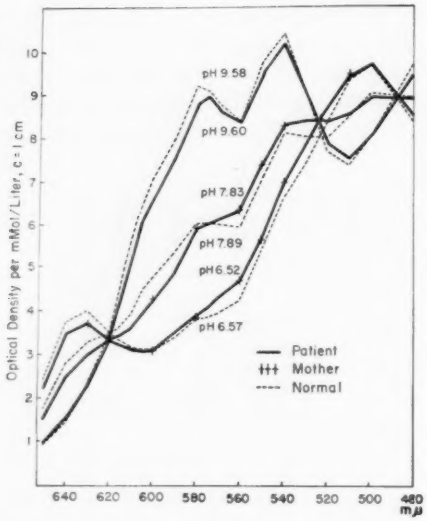

FIG. 6. ABSORPTION SPECTRA OF METHEMOGLOBIN. pH as indicated; phosphate or borate buffer of 0.1 ionic strength.

510 mμ region where both oxyhemoglobin and hemoglobin have lower light absorptions than has methemoglobin. Furthermore, mathematical analysis of the curves at several pH's indicates that if the curves represent mixtures, the light absorption of both components must be highly pH-dependent, which is not the case in ferro-hemoglobins. The Soret peak of the patient's methemoglobin was at 408 mμ as compared with 406 mμ in methemoglobin A. The described spectral abnormalities were readily reproducible. They did not change on addition of excess ferricyanide or after standing several days. They are considered to be a manifestation of abnormal molecular structure of the abnormal pigment. To allow absolute comparisons, all absorption spectra are presented as millimolar densities which refer to the total pigment concentration and not necessarily to the concentration of the abnormal pigment.

The dissociation of methemoglobin ($Fe^{+++}OH_2$) into the alkaline form ($Fe^{+++}OH$) is demonstrated in Figure 7. The difference in the pK between normal and the patient's pigment is of doubtful significance. The oxidized pigment of the patient reacted quantitatively with potassium cyanide, and the resultant cyanmethemoglobin exhibited essentially normal absorption spectra.

Electrophoretic findings. Starch block electrophoresis of the oxyhemoglobin, carboxyhemoglobin and cyanmethemoglobin preparations did not reveal significant differences in the migration of the patient's pigment in comparison with hemoglobin A, except for a very light A_2 component in the patient. On starch block electrophoresis of the methemoglobin form at pH 8.6, patterns were obtained which were identical in the patient and his mother and which could easily be distinguished from methemoglobin A (Figure 8). The bulk of the patient's pigment migrated more slowly than methemoglobin A, but it is difficult to decide whether the spot of the latter was entirely vacated. Eluates from this portion gave absorption spectra identical with the main band, and both were indistinguishable from the absorption spectrum of the pigment before electrophoresis. At pH 7.05 and at 6.5 the patient's methemoglobin migrated more slowly toward the cathode than did methemoglobin A. The dissociation of the $Fe^{+++}OH_2$ was not significantly different in the patient's methemoglobin (Figure 7) and the anomalous change in the net charges at alkaline pH therefore reflects differences in the pK of other groups.

DISCUSSION

The lack of hypoxic manifestations in the intensely cyanotic patient is explained by the shift of the entire oxyhemoglobin dissociation curve (Figure 1). Assuming a normal tissue P_{O_2} of 35 mm Hg and disregarding the Bohr effect, normal blood can dissociate from 95 per cent arterial saturation to 60 per cent oxyhemoglobin in the

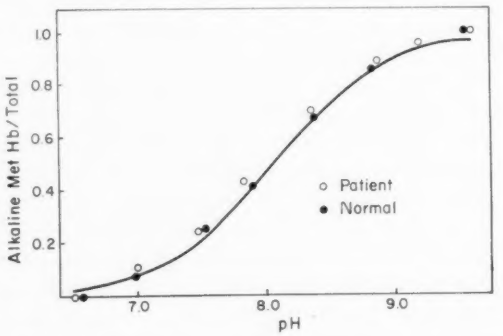

FIG. 7. DISSOCIATION OF METHEMOGLOBIN. The solid line describes the theoretical dissociation with a pK of 8.06. Circles are the observed values corrected to ionic strength of 0.10.

tissue capillaries. The patient's arterial blood was only 60 per cent saturated, but it dissociated to 25 per cent oxyhemoglobin at the same tissue tension. It thus delivers, like normal blood, 35 per cent of its oxygen capacity at peripheral oxygen tensions of 35 mm Hg.

The observed low oxygen affinity of the hemoglobin in buffered solutions leaves little doubt that the abnormal dissociation curve of the blood was caused by the properties of the hemoglobin and not by an abnormal environment within the red cell. Abnormal dissociation curves of the blood from subjects suffering from hemoglobin S disease have been reported (12, 13). The inherent oxygen affinity of hemoglobin S after dialysis was found to be normal, and the abnormal oxygen affinity of the blood has been attributed to dialyzable constituents within the red cell. The buffered and dialyzed hemoglobin solutions from our patients showed lowered oxygen affinities proportional to those found in their whole blood.

Numerous spectroscopic examinations of the native blood as well as of the hemoglobin solutions failed to detect any pigment with spectral characteristics other than those of oxyhemoglobin and hemoglobin. The patient's arterial blood contained 60 per cent oxyhemoglobin. If a significant portion of the remaining 40 per cent were different from reduced hemoglobin, it should have been detectable in spite of the limited accuracy of spectrophotometric analysis of systems with more than two components.

A shift in the peak of the Soret band to shorter wave length is, according to Jope (14), one of the most sensitive criteria of denaturation of a hemoglobin. The Soret peak of the oxyhemoglobin from the patient was precisely at 415 mμ and that of his hemoglobin at 425 mμ. Denaturation can not be incriminated, therefore, as the cause of the abnormal oxygen affinity. Although it can be influenced by major changes in the protein carrier, the absorption spectrum in the spectral region between 700 and 350 mμ is primarily a reflection of the configuration of the hemes. The quantitatively normal absorption spectra of both the oxyhemoglobin and the hemoglobin form of the abnormal pigment in this spectral region lends no support to any abnormality in the heme groups. These findings in conjunction with the anomalous electrophoretic mobility implicate the amino acid

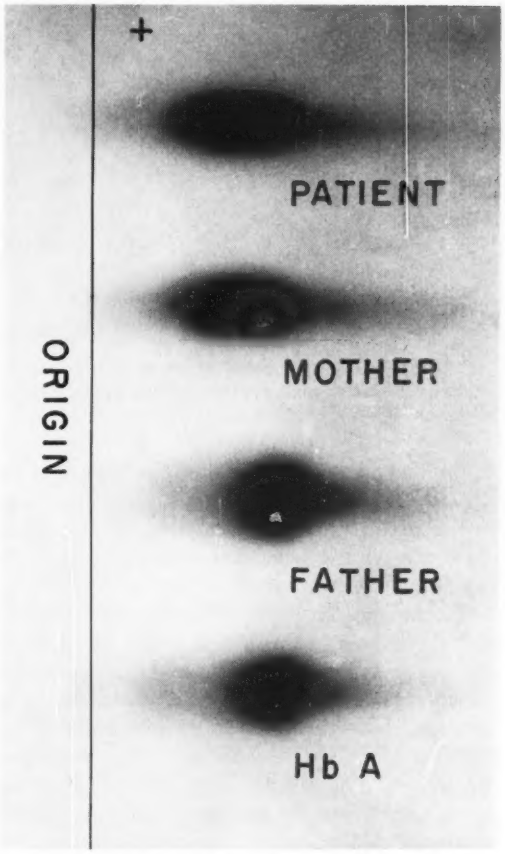

FIG. 8. STARCH BLOCK ELECTROPHORESIS OF METHEMOGLOBIN. Veronal buffer; 0.05 ionic strength, pH 8.6, 250 v, 40 ma, 16 hours.

chains as the probable site of the abnormality. The relatively slight alteration in electrophoretic mobility in no way conflicts with the large abnormality of the oxygen affinity. The former depends upon net electrical charges in the protein molecule and does not necessarily reflect structural abnormalities which alter oxygen affinity.

The shape of the oxyhemoglobin dissociation curve deserves some comment. In the semilogarithmic presentation (Figure 2) factors like pH and temperature, which affect oxygen affinity, merely displace the curve along the abscissa but they do not alter its shape. The steepness of the normal curve is attributed to the heme-heme interactions in the sense that the oxygenation of one or more of the hemes facilitates the oxygenation

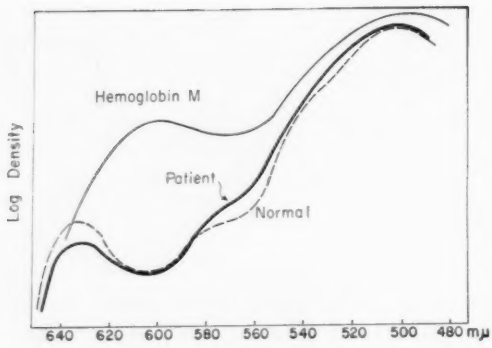

FIG. 9. COMPARISON OF THE SPECTRAL ABNORMALITIES OF THE PATIENT'S OXIDIZED HEMOGLOBIN WITH METHEMOGLOBIN M (21) AT pH 6.5.

of the remaining hemes in the same molecule (1, 2). It is convenient to assign a numerical value to the intensity of this interaction by calculating the *n* value in the logarithmic form of Hill's equation, although it is realized that the equation no longer has precise physical meaning as originally thought. An *n* value of 1 is found in pigments without interactions, for instance in myoglobin which contains only 1 heme per molecule. The abnormal hemoglobin exhibited an *n* value of 1.1 versus 2.6 in the normal control. One is tempted to interpret this finding as the absence of heme-heme interactions in the abnormal hemoglobin; however, uncertainties arise from the lack of unequivocal electrophoretic isolation of the abnormal hemoglobin. The hemolysate very likely contained unknown amounts of a normally reactive hemoglobin. It is theoretically possible that the flat oxyhemoglobin dissociation curve in Figure 2 is the resultant of the curve of the normal fraction plus an equally steep curve of the abnormal fraction which lies considerably to the right of the normal curve.

Riggs (15) has reported a large though not complete blocking of the heme interactions resulting from blocking of the sulfhydryl groups of normal hemoglobin by means of *p*-chloromercuribenzoate. The *n* value dropped from 2.9 to 1.4, and the effect could be reversed largely by glutathione. Addition of glutathione has not changed the oxygen equilibria of the patient's hemoglobin, but this negative result permits no conclusion regarding an intrinsic abnormality of the sulfhydryl groups.

The described hemoglobin shows some similarities to hemoglobin M, viz., it causes cyanosis, its oxidized form exhibits spectral abnormalities, and electrophoretic abnormalities can best be demonstrated in its met form. In some cases of hemoglobin M, including the original case of Hörlein and Weber (16), up to 30 per cent methemoglobin of abnormal spectral characteristics was present in the native blood and accounted for the cyanosis (17, 18). In the case reported by Kiese, Kurz and Schneider (19), enzymatic reduction of this methemoglobin fraction yielded a hemoglobin with normal oxygen affinity. If these findings apply generally to hemoglobin M, the primary defect in its oxygen transport function would be its tendency to become oxidized due to structural abnormalities in the molecule, a mechanism quite different from the truly diminished oxygen affinity in our abnormal hemoglobin. In other cases classified as hemoglobin M (20, 21), including a large number reported in Japan (22), no oxidized pigment could be detected in the blood. Only *in vitro* oxidation produced a fraction of the methemoglobin with abnormal spectral characteristics similar to those found in the first-mentioned group. No oxygen equilibria have been reported in these cases, but it is assumed that the pigment present in these cyanotic patients was unable to combine reversibly with oxygen. A comparison of the spectral abnormalities of the met form of hemoglobin M and of the pigment reported here is given in Figure 9. Since the absolute absorption of both abnormal pigments is unknown, the absorption spectra are presented as the logarithm of optical densities in order to make the shape of the curve independent of concentration. The rather different spectral abnormalities very likely reflect different molecular structure, which is also manifested by their differences in reactivity toward oxygen.

In spite of these significant differences, the various types of hemoglobin M and the pigment reported here may belong to the same functional group of abnormal hemoglobins, namely those with aberrations in amino acid groups which are crucial in the interaction between globin and the oxygen-combining centers. Several hypothetical explanations of the normal interactions have been advanced, especially in connection with the Bohr effect and the heme-heme interactions (2). The

chemical nature of the groups involved, even that of the heme-linked acid groups, is still an open question (1). It would be premature therefore to speculate on the globin anomalies of the described hemoglobin. It is to be expected that future elucidation of amino acid anomalies in this now emerging group of hemoglobins with altered oxygen affinity may shed some light on the nature of the normal heme-globin interactions.

SUMMARY

A familial human hemoglobin variant is described which caused markedly diminished oxygen saturation of the blood in the presence of normal arterial oxygen tensions. The shift of the entire oxyhemoglobin dissociation curve was due to an abnormally low oxygen affinity of the hemoglobin. The oxygen equilibria of the pigment in buffer solutions revealed a Bohr effect of normal magnitude but probably no heme-heme interactions. The absorption spectra of its oxy and reduced form in the visible and ultraviolet spectral region were those of normal hemoglobin. The alkaline and acid methemoglobin showed spectral abnormalities. On starch block electrophoresis, the methemoglobin form of the pigment migrated more slowly than methemoglobin A. The lowered oxygen affinity is attributed to molecular aberrations in the globin which interfere with normal heme-globin interactions.

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THE INCORPORATION OF COPPER INTO CERULOPLASMIN *IN VIVO*: STUDIES WITH COPPER^{64} AND COPPER^{67} *

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The fact that absence or deficiency of the plasma copper-protein, ceruloplasmin, is almost always associated with the excessive deposits of tissue copper which characterize Wilson's disease has suggested that ceruloplasmin regulates copper absorption or excretion (1). Based on the *in vitro* demonstration of reversible dissociation of copper from ceruloplasmin, a hypothesis was proposed of how such regulation could be effected (2). This stated that if reversible dissociation of copper from ceruloplasmin occurred in the intestinal wall *in vivo*, a concentration of copper ions would result which could oppose the net transfer of copper ions from the intestinal lumen into the blood. The three experiments reported in this paper were designed to determine whether exchange of ceruloplasmin copper for ionic copper, which is equivalent to reversible dissociation of ceruloplasmin copper, takes place *in vivo*.

In the first experiment, exchange of ceruloplasmin copper was looked for in patients with Wilson's disease in whom virtually no synthesis of the protein was occurring. The interpretation of this experiment required knowledge of whether the catabolism of ceruloplasmin was detectably different in a normal individual from its catabolism in patients with Wilson's disease. A comparison of the half-life of ceruloplasmin, as one measure of its catabolism, was made in these two types of individuals in the second experiment. In the third experiment, the incorporation of ionic copper into ceruloplasmin was studied in an individual with normal copper metabolism in such a way that incorporation occurring during synthesis of ceruloplasmin could be differentiated from that resulting from copper exchange.

All of our results were compatible with the conclusions that copper entered the ceruloplasmin molecule only at the time of synthesis, and that exchange of ceruloplasmin copper with ionic copper did not occur *in vivo*. Since it was a corollary of these conclusions that a given copper atom was a label which remained on a ceruloplasmin molecule throughout its life span, we were able to calculate the rate of turnover of ceruloplasmin, and its copper, in an individual with normal copper metabolism.

EXPERIMENTAL

Materials and methods

Ceruloplasmin. Two solutions (CH-157 and CH-240), which contained 2.9 and 2.1 g of purified human ceruloplasmin per 100 ml, respectively, were obtained from the Ortho Research Foundation, Raritan, N. J.[1] Both preparations were sterile, nontoxic, nonpyrogenic and their intravenous administration to human beings produced no ill effects.

Copper^{64}. Sterile solutions of cupric^{64} acetate were obtained from Abbott Laboratories, Oak Ridge, Tenn. The half-life of this isotope is 12.8 hours (3).

Copper^{67}. Copper^{67} was produced in the 60-inch cyclotron of the Brookhaven National Laboratory by the following (α, p) reaction: $Ni^{64} + He^4 \rightarrow Cu^{67} + H^1$. Since there is only 1 per cent of Ni^{64} in naturally occurring nickel, material enriched to 96 per cent Ni^{64} was obtained from the Isotope Division of the Oak Ridge National

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[1] Through the cooperation of Sam T. Gibson, Director, American National Red Cross Blood Program, and James A. McComb, John M. Newell and Lewis H. Larsen of the Biologic Laboratories, Department of Public Health of the Commonwealth of Massachusetts, we obtained Cohn fraction IV-1 from human plasma. Ceruloplasmin was prepared from fraction IV-1 under the direction of H. O. Singher of the Ortho Research Foundation, Raritan, N. J.

TABLE I

Labeling of ceruloplasmin with copper^{67} by Method A

Expt.	Subj.	Ceruloplasmin (CH-240)		0.85% NaCl	Ascorbic acid	Cu^{67}-nitrate*	Cu^{67}	Final pH
		ml	*g*	*ml*	*mg*	*ml*	*μg*	
I	G.G.	63.0	1.32	240	785	2.3	71	6.18
I	D.S.	63.0	1.32	340	785	2.0	62	6.45

* This solution contained 31 μg copper/ml with a specific activity of 1.21 μc/μg.

Laboratory and used as the target. Although enriched nickel^{64} was relatively expensive, it could be recovered with a yield of about 98 per cent and reused.

To prepare approximately 1 to 2 mc of copper^{67}, 60 mg of the nickel^{64} was electroplated as metal onto gold foil that had been silver-soldered to a copper target holder. (The area covered by the nickel was roughly $\frac{1}{4} \times \frac{3}{8}$ inch. It varied in thickness from 0.007 to 0.010 inch, which was sufficient to absorb the entire alpha beam.) The target assembly was mounted on a water-cooled probe that was inserted into the vacuum chamber of the cyclotron, and the nickel^{64} target was bombarded by the internal beam of 40 Mev alpha particles. At the current of 50 to 60 μa which resulted, bombardments of 10 to 12 hours were required. For example, 1.4 mc was produced in a 500 μa-hour bombardment, which corresponded to a thick-target yield of 2.8 μc per μa-hour.

The target was removed from the cyclotron and the gold foil was separated from the target holder by melting the silver solder. The bombarded nickel was dissolved in a few drops of nitric acid. After addition of 0.5 mg of copper carrier, hold-back carriers of indium, cadmium and cobalt, and 2 or 3 drops of concentrated sulfuric acid, the copper was electroplated at 80° C for 30 minutes onto a platinum electrode. The copper was dissolved from the platinum with nitric acid and was used without sterilization. The processing yield was estimated to be at least 95 per cent. The only radiocontaminant present in significant amount was copper^{64}, which constituted initially about 7 per cent of the total radioactivity.

Nitric acid was removed from the solution which remained after the electroplating of copper by evaporation to fumes of sulfur trioxide. The residue was diluted with water, made alkaline with concentrated ammonia, and the nickel^{64} was recovered by electroplating.

The half-life of copper^{67} was determined to be 61.8 ± 0.8 hours. To our knowledge the experiments reported in this paper represent the first use of this isotope in biological research.

Radioactive labeling of ceruloplasmin copper

1. Method A. To a solution of purified ceruloplasmin in physiological saline, ascorbic acid and then copper^{67} nitrate were added. After standing at room temperature for 1.5 hours, approximately 35 per cent of the copper^{67} was found to have been incorporated into the ceruloplasmin of each solution by exchange. The amounts of material used for the two preparations are given in Table I.

2. Method B. Two male volunteers, aged 50 and 64, received 1.0 mg of ethinyl estradiol (Estinyl) daily for 5 days in order to elevate their plasma concentrations of ceruloplasmin (4, 5). By intravenous injection they were then given, respectively, 3.45 and 4.77 mg of Cu^{67}, with a specific activity of 105 μc per mg, as cupric^{67} nitrate.[2] Forty hours later a phlebotomy was performed on each volunteer yielding, in a total of 450 ml of plasma, 286 mg of labeled ceruloplasmin. This contained 88 μg of ceruloplasmin-bound Cu^{67}, and only 1.3 μg of nonceruloplasmin Cu^{67}. Thus, this material was of much greater value in studying ceruloplasmin metabolism in a control subject than ceruloplasmin labeled by Method A which always contained a large amount of nonceruloplasmin copper^{67}. Although the incidental administration of the latter was unimportant in the experiment in the 2 patients with Wilson's disease, who cannot synthesize ceruloplasmin, nonceruloplasmin copper would have entered newly synthesized ceruloplasmin in control subjects *in vivo* and made Experiment II impossible.

Quantitative determination of ceruloplasmin. Ceruloplasmin concentrations in serum were measured by determining the oxidase activity of serum toward paraphenylenediamine (2).

Quantitative determination of radioactive copper^{64} and copper^{67}. Four-ml aliquots of serum, or smaller aliquots brought to 4 ml by addition of normal saline, were counted in a Nuclear-Chicago DS5-5 well-type scintillation counter by means of a 161A scaler. Discrimination between the 0.184 Mev gamma rays of Cu^{67} (3) and the 0.51 Mev annihilation gamma rays (6) of Cu^{64} was effected by inclusion of a Nuclear-Chicago 1810 radiation analyzer in the circuit. Correction for background and physical decay yielded net corrected counts per minute per milliliter of serum due to each isotope.

Separation of nonceruloplasmin copper. In order to determine the proportion of the radioisotopic copper in serum bound to ceruloplasmin in Experiments I and III, the nonceruloplasmin copper in the serum was made to react with sodium diethyldithiocarbamate (DTC) and

[2] In order to keep the copper in solution, each dose of Cu^{67} was dissolved in a mixture of 60 ml Amigen (5 per cent enzymic casein hydrolysate and 5 per cent dextrose, in water) and 0.5 ml of 7.5 per cent sodium bicarbonate. The solutions were clear, blue, and had a pH of 5.2. Neither produced any untoward reaction when infused.

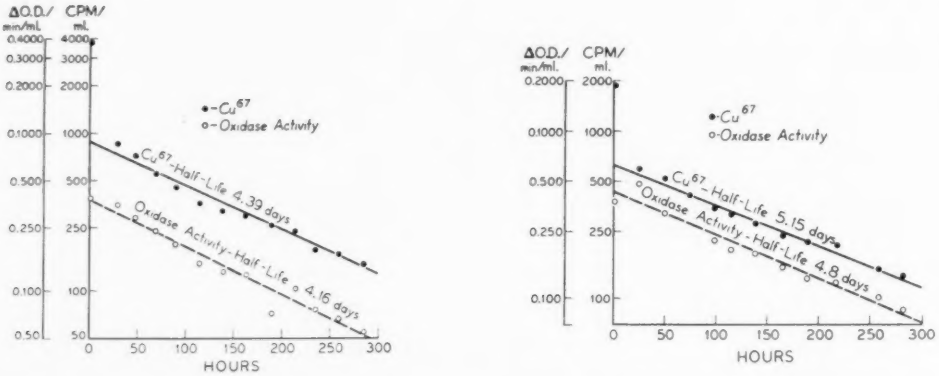

FIG. 1. HALF-LIFE OF COPPER^{67}-LABELED CERULOPLASMIN OBTAINED FROM THE RATES OF DISAPPEARANCE OF OXIDASE ACTIVITY AND RADIOACTIVITY OF SERUM IN TWO ACERULOPLASMINEMIC PATIENTS WITH WILSON'S DISEASE, G.G. (LEFT) AND D.S. (RIGHT).

the colloidal Cu-DTC complex was adsorbed on a column of activated charcoal, as follows. After dilution of 3 ml of serum with an equal volume of normal saline, 0.6 ml of a 0.1 per cent solution of DTC in saline was added. The mixture was passed through a column of 100 mg of activated charcoal (Norit-A). The 4 ml of effluent which followed the 1.5 ml fore-run had the same concentration of ceruloplasmin as that contained in the 6.6 ml of solution passed over the column, and contained neither free copper nor free DTC.

RESULTS

Experiment I. We infused 1.32 g of ceruloplasmin, labeled by Method A with copper^{67}, into each of two patients with Wilson's disease without serum ceruloplasmin. There were no untoward effects on pulse, respiration, blood pressure, or venous clotting and prothrombin times. Serial measurements of the serum concentration of the protein were obtained both by *1*) determining the oxidase activity of the serum toward paraphenylenediamine and *2*) counting the radioactivity of the serum. The latter was approximately equivalent to determining copper^{67} in ceruloplasmin since, beyond 24 hours after infusion, less than 10 per cent of copper^{67} in serum was non-ceruloplasmin copper^{67}. The results are given in Figure 1. The values for the half-life of ceruloplasmin given in the figure and in Table II were calculated independently from the results of both the enzymatic and radioactive measurements by the method of least squares. The volumes of distribution of the infused protein (Table II) were obtained by dividing the amounts administered by the serum concentration of ceruloplasmin at zero time determined by extrapolation.

TABLE II

Volumes of distribution, half-lives and daily turnover rate of ceruloplasmin and ceruloplasmin copper

Expt.	Subj.	Volume of distribution	Oxidase activity half-life	Radioactivity half-life	Daily turnover rate of ceruloplasmin*		Daily turnover rate of ceruloplasmin copper
		L	*days*	*days*	*%*	*mg*	*mg*
I	G.G. (Wilson's disease)	3.77	4.16	4.39	15.7	207.2†	0.704
I	D.S. (Wilson's disease)	3.41	4.80	5.15	13.4	176.9†	0.601
II	J.M. (control)	3.97		5.40	12.9	141.9	0.483

* Calculated from radioactivity measurements.
† Immediately after infusion.

Experiment II. Into a normal male volunteer, aged 22, 450 ml of plasma containing 286 mg of copper^{67}-labeled ceruloplasmin (Method B) was infused. The concentration of the copper^{67}-labeled ceruloplasmin was measured daily by determining the radioactivity of whole serum, and these results were used to calculate the half-life of ceruloplasmin and its volume of distribution (Figure 2 and Table II). In contrast to the previous experiment, we could not also calculate these values from measurements of oxidase activity, since the oxidase activity of the infused, labeled ceruloplasmin could not be distinguished from that of the subject's own ceruloplasmin which was continually being synthesized.

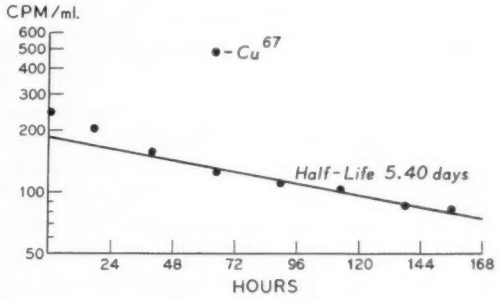

FIG. 2. HALF-LIFE OF COPPER^{67}-LABELED CERULOPLASMIN OBTAINED FROM THE RATE OF DISAPPEARANCE OF THE RADIOACTIVITY OF SERUM IN A CONTROL SUBJECT (J.M.).

Experiment III. A 39 year old female with Huntington's chorea and no detectable abnormality of copper metabolism was given 2 mg of copper^{64} orally, as cupric^{64} acetate, on three different and widely separated occasions. *1)* The first dose was administered when the patient's ceruloplasmin concentration was at a normal value, 26.7 mg per 100 ml of serum. *2)* The second was given 5 days after she had received, for 15 days, 1 mg ethinyl estradiol daily. The estrogen served to increase the rate of synthesis of ceruloplasmin and to raise its serum concentration from 29.2 to 51.9 mg per 100 ml (4, 5). *3)* The third administration of copper^{64} occurred 1 day after she had received an infusion of 1.39 g of purified human ceruloplasmin (CH-157). The infusion did not, of course, increase the rate of synthesis of ceruloplasmin, but did raise its serum concentration from 25.5 to 74.1 mg per 100 ml. The concentration of serum copper^{64} and its proportion in ceruloplasmin were measured at intervals (Figure 3) after each dose of copper^{64}.

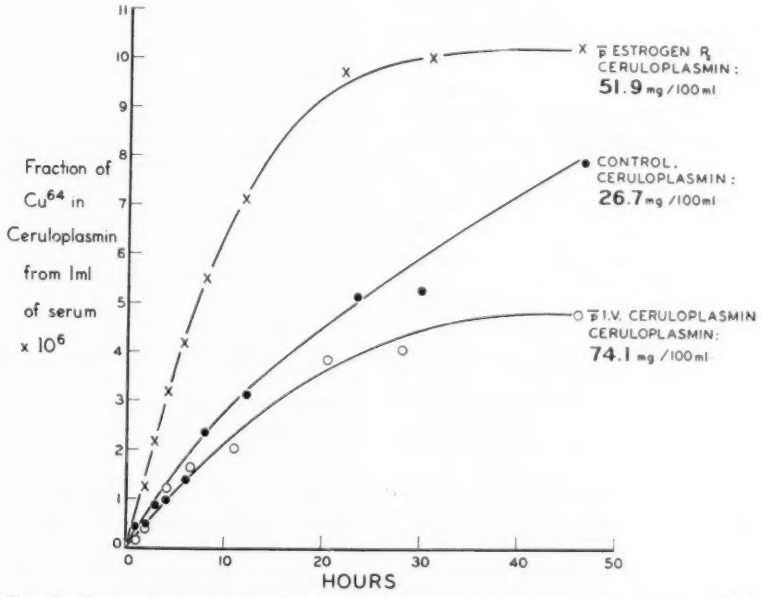

FIG. 3. INCORPORATION OF COPPER^{64} INTO CERULOPLASMIN IN A CONTROL SUBJECT (P.N.) UNDER THREE DIFFERENT EXPERIMENTAL CONDITIONS.

DISCUSSION

Experiment I indicated that *in vivo* exchange of ceruloplasmin copper did not occur in either of two patients with Wilson's disease. Entire ceruloplasmin molecules disappeared at a rate not significantly different from that at which ceruloplasmin-copper^{67} disappeared from the plasma.[3] The latter rate should have exceeded the former if exchange had occured between ceruloplasmin-copper^{67} and the "cold" copper of the body.

It is, of course, conceivable that the ceruloplasmin which we infused could have been altered during fractionation, in which case the absence of exchange might only imply that this ceruloplasmin had lost its physiological integrity. Yet by every other property which was measured the ceruloplasmin seemed undenatured: its labile blue color (7), its tight copper-binding, its electrophoretic mobility (7), its lack of antigenicity in human beings,[4] and its oxidase activity (10) were virtually identical with these properties of ceruloplasmin in whole plasma or serum. Nevertheless, this experiment did not provide evidence that exchange might not have occurred in normal individuals, since the metabolism of ceruloplasmin may differ in the two groups. Consequently, we performed Experiment II in order to compare the half-life of ceruloplasmin, as measured in these patients with Wilson's disease, with the half-life in a normal individual. The half-life of copper^{67}-labeled ceruloplasmin did not differ significantly in a simultaneous test of the values determined in the two patients and in the control subject.[5] By this criterion, therefore, ceruloplasmin did not seem to be metabolized at a different rate in the patients than in the control subject.

Rather similar values for the half-life of ceruloplasmin in patients with Wilson's disease and in control individuals, whether determined in studies with purified ceruloplasmin or with whole blood, have also been found previously. Thus we have reported the half-life of ceruloplasmin to be 3.2 to 4.7 days after the infusion of whole blood (8), and 5.6 to 7.2 days after administration of unlabeled purified human ceruloplasmin (9), in patients with Wilson's disease. Gitlin and Hughes [quoted in (12)], using I^{131}-labeled purified human ceruloplasmin, found half-lives of 5 to 7 days in five chronically ill subjects without Wilson's disease.[6] Half-lives of fibrinogen (13, 14), the γ-globulins (12), and albumin (15) have also been compared in subjects with hereditary deficiency of the protein and in control subjects. The results do not consistently indicate either similar or different half-lives for these proteins in the two kinds of individuals.

Finally, Experiment III confirmed that *in vivo* exchange did not occur in an individual without an apparent abnormality of copper metabolism. When P.N.'s rate of synthesis of ceruloplasmin was approximately doubled by the administration of estrogen (Study B), about twice as much copper^{64} was incorporated into the protein as in control Study A. However, when an even higher serum concentration of ceruloplasmin was achieved passively, that is, by infusion (Study C), the incorporation of copper^{64} into ceruloplasmin was essentially the same as that which occurred in Study A, indicating that exchange was not occurring.

Our inability to demonstrate exchange of ceru-

[3] For each patient the differences between the log (oxidase activity) and log (radioactivity) at each sampling time were plotted against time, the slope of the resulting line being designated as b. Then $|b-0|/SE_b = |t|$ was calculated, for the indicated degrees of freedom, and found to be $t_{10} = 0.785$ for G.G. and $t_8 = 1.2104$ for D.S. In both cases a $|t|$ as great, or greater, might have been found, by chance alone, more than 50 per cent of the time.

[4] Insofar as this is indicated by the constancy of the determined half-life and the lack of any clinical reaction incident to repeated infusions of purified ceruloplasmin in G.G. and D.S. over the past 4 years (8, 9).

[5] An analysis of covariance (11) was used to test the hypothesis that these three values for the half-life did not differ significantly. An F of 3.129, with 2 and 23 degrees of freedom, was obtained which could occur by chance alone more than 5 per cent of the time.

[6] Differences in the values of half-lives may reflect the manner in which they are calculated. Thus, our values in Experiments I and II are calculated from all of the points determined after the first 24 hours. If, however, we assume that 3 days is required for equilibration of infused ceruloplasmin between plasma and extravascular compartments, the results are somewhat different. For example, the latter assumption yields 4.80, 5.97 and 6.36 days for the three values which were given in Table II as 4.39, 5.15 and 5.40 days, respectively. For the purposes of the present paper, the important point is that all values of a half-life which are to be compared should be calculated from measurements made after the same period of equilibration.

loplasmin copper for ionic copper in any of these experiments is strong evidence that reversible dissociation of copper from ceruloplasmin does not occur *in vivo*. Since the hypothesis which two of us have advanced [I.H.S. and A.G.M (2)] and which was briefly outlined above, was predicated on the assumption that such dissociation does occur, this hypothesis is not likely to be true.

If, however, a copper atom labels a ceruloplasmin molecule throughout its lifetime we can use our results to calculate the volume of distribution and turnover of ceruloplasmin. The values for volume of distribution are remarkably similar in all three subjects (Table II). The percentage turnover of ceruloplasmin and of ceruloplasmin copper was calculated in all three subjects from the standard equation (16) (Table II), and the fact that the copper content of the protein is 0.34 per cent (7). The value of 142 mg for the daily turnover of ceruloplasmin in the control subject, J.M., corresponds to 0.5 mg of copper incorporated into ceruloplasmin daily. This must be close to the amount of copper which is absorbed from the 2 to 5 mg of copper ingested in the diet (17). Thus it seems that much of the absorbed dietary copper in a normal individual may ultimately be bound to a ceruloplasmin molecule of 151,000 mol wt. Such bound copper should be confined largely to the vascular compartment, and the volume of distribution of 3.97 L found in J.M. indicates that it is.

Despite the apparent congruence of the amount of copper absorbed and that which is used daily for the synthesis of ceruloplasmin, our results do not greatly clarify the physiological role of ceruloplasmin and its relationship to Wilson's disease. They yield no information, and little additional data are available, about: *1*) the period which elapses between absorption of dietary copper and its quantitative incorporation into ceruloplasmin; *2*) the fate of copper during this time; and *3*) whether, and where, ceruloplasmin copper is excreted. Nor do they elucidate the significance of patients with Wilson's disease who have normal serum concentrations of ceruloplasmin, or of healthy heterozygous carriers of the disease who have abnormally low serum concentrations of this protein (1, 18). Furthermore, the physiological significance of the *in vitro* demonstrations of the reversible dissociation of ceruloplasmin copper (2, 19), of the oxidase activity of ceruloplasmin (1) and of its heterogeneity (20) is also obscure. These questions require further investigation.

SUMMARY

1. The hypothesis that ceruloplasmin exchanges its copper for ionic copper *in vivo* was tested, by means of ceruloplasmin labeled with copper^{64} and copper^{67}, in two patients with Wilson's disease and in two control subjects.

2. No indication of exchange was found in any of three different experiments. It appears that copper is incorporated into the ceruloplasmin molecule *in vivo* only at the time of synthesis of the protein and not through exchange.

3. A corollary of these results is that copper is a lifelong label of a ceruloplasmin molecule so that radioactive copper may be used for the determination of the turnover of ceruloplasmin and ceruloplasmin copper.

4. The amount of copper incorporated daily into ceruloplasmin in a control subject corresponds closely to the amount of copper absorbed from the dietary intake.

ACKNOWLEDGMENTS

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LOW LEUKOCYTE PHOSPHORYLASE IN HEPATIC PHOSPHORYLASE-DEFICIENT GLYCOGEN STORAGE DISEASE

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Glycogen storage disease was first recognized as a clinical entity by Von Gierke in 1929 (1). Subsequently numerous clinical and biochemical studies have indicated that this syndrome is composed of several distinct entities (2, 3). The present-day classification of glycogen storage disease into five subgroups is based on the clinical picture, and in some, on the demonstration of specific enzymatic defects (4). The most common type of the disease (Von Gierke's disease) results from the lack of glucose-6-phosphatase in the liver (5). The term Pompe's disease is used to denote a group of children with generalized glycogen deposition, especially in the heart, who usually die before 8 months of age of cardiac complications (6). The third type of the disease represents cases in which debranching enzyme has been low in the livers of the patients studied (7). In the fourth type, branching enzyme is thought to be deficient on the basis of abnormal glycogen structure (8), but no definite assay has been performed to confirm this. Under the fifth subgroup, two separate syndromes have been described—patients with a progressive myopathy and absent muscle phosphorylase (9, 10), and patients with hepatomegaly and low liver phosphorylase (11).

In 1959 Hers (11) presented data on three patients with glycogen storage disease who had low levels of liver phosphorylase, but normal levels of glucose-6-phosphatase and amylo-1,6-glucosidase; muscle phosphorylase was normal. Recently we have studied two brothers with glycogen storage disease who were also found to have low liver phosphorylase but normal glycogen structure and glucose-6-phosphatase activity. The clinical data on these two patients will be presented in detail elsewhere (12).

In view of the observation that leukocyte glycogen is elevated in glycogen storage disease (13) and the desirability of establishing the nature of the enzymatic defect without resorting to liver biopsy, methods were devised to measure the activities of leukocyte phosphorylase and glucose-6-phosphatase. These were then applied to the above two patients, their relatives and a group of control subjects. The results of this study form the basis of this report.

MATERIALS AND METHODS

I. Biochemical studies. The two patients studied were brothers, aged 7 and 9, who had enlarged livers, with increased glycogen content but normal glycogen structure and glucose-6-phosphatase activity. Fasting blood sugars were somewhat low but the hyperglycemic response to epinephrine and glucagon was adequate in both. Muscle glycogen was normal. Liver phosphorylase was found to be low in both patients when assayed on specimens obtained by open liver biopsy.[1] The remaining family members included the father, aged 38, mother, aged 37, brother, aged 12, and sister, aged 11. In none of them was there a history of liver disease nor was hepatomegaly found on examination.

II. Leukocyte studies. Twenty ml of blood was drawn by venipuncture from patients in the fasting state. White blood cells were obtained by the fibrinogen sedimentation technique (14) using 3 per cent bovine fibrinogen in Krebs-Ringer bicarbonate buffer (pH 7.4). The total number of leukocytes was estimated in the supernatant after a 20-minute sedimentation. After centrifugation of the supernatant at 755 G for 20 minutes, the button of cells, which contained approximately a 1:1 ratio of leukocytes to erythrocytes, was suspended in 1 ml of NaF (0.1 M). After homogenization by ultrasonication (10 kc) for 2 minutes, phosphorylase activity was determined by the method of Sutherland (15). Three-tenths ml of the sonicate was added to tubes containing 0.1 ml of 0.02 M adenosine-5'-phosphate and 0.1 ml water. At zero time, 1 ml of a glucose-1-phosphate solution (0.05 M, pH 6.1) containing 5.7 mg glycogen per ml and NaF (0.05 M) was added. Immediately, a 0.5 ml aliquot was added to 2 ml of 10 per cent trichloroacetic acid, and inorganic phosphorus was determined by the method of Fiske and Subbarow (16). The tubes were then incubated for 30 minutes at 37° C in a Dubnoff

[1] We are indebted to Dr. Barbara Illingworth Brown, Washington University of St. Louis, for performing the studies on the liver biopsy specimens.

TABLE I

Leukocyte phosphorylase and glycogen levels in patients with glycogen storage disease and their family

Patient	Age	Relation	Condition	Glycogen	Phosphorylase
				μg/10⁷ leukocytes	*μg P liberated/10⁷ WBC*
Jo.F.A.	38	Father	Normal		33.2
A.A.	37	Mother	Normal		10.2*
Ja.A.	12	Son	Normal		33.6
R.A.	10	Son	Affected	35.4	7.1*
Jo.S.A.	7	Son	Affected	29.2	5.5*
Normal (20)†				34.3 ± 0.9‡	29.6 ± 1.2‡

* Average of four separate determinations.
† Number of control subjects.
‡ Standard error.

metabolic shaker. At the end of incubation another 0.5 ml aliquot was removed for inorganic phosphorus determination. Appropriate blanks without glucose-1-phosphate and without sonicate were included in each experiment. Results were expressed as micrograms of inorganic phosphorus liberated per 10^7 leukocytes per 30-minute incubation.

Glycogen was determined by the method of Carroll, Longley and Roe (17) on the leukocyte button suspended in 3 ml of 5 per cent trichloroacetic acid. After sonication for 2 minutes the solution was filtered through acid-washed filter paper. To 1 ml of this filtrate was added 5 ml of 99 per cent ethanol, and glycogen precipitation was allowed to occur overnight at room temperature. The precipitate was centrifuged at 1,475 G for 10 minutes and the supernatant carefully decanted. The pellet was dissolved in 2 ml of water and glycogen was determined with anthrone. Results were expressed as micrograms of glycogen per 10^7 leukocytes. Glucose-6-phosphatase was determined in sonicated leukocytes, using the method of Swanson (18).

The activation of phosphorylase by glucagon was studied by the method of Sutherland and Cori (19). The leukocyte-rich supernatant solution obtained from sedimentation with 3 per cent fibrinogen (usually about 20 ml) was preincubated in 50-ml siliconized flasks in an atmsophere of 95 per cent O_2 and 5 per cent CO_2 for 30 minutes in a Dubnoff metabolic shaker at 37° C. At the end of this incubation 0.1 mmole of glucagon was added to the appropriate flasks and incubation continued for another 30 minutes. Equal amounts of buffer without glucagon were added to appropriate flasks which served as controls. Phosphorylase activity of the leukocytes was then determined as described above.

Bovine fibrinogen was obtained from Armour Pharmaceutical Company. Glycogen was purchased from

TABLE II

Leukocyte phosphorylase activity in control subjects

Patient	Age	Sex	Condition	Leukocyte phosphorylase activity
				μg P liberated/10⁷ WBC
P.D.	17	F	Normal	30.8
E.M.	18	F	Normal	20.6
C.G.	35	M	Normal	21.4
K.S.	20	F	Normal	32.5
P.M.	18	F	Normal	28.8
H.W.	28	M	Normal	31.0
S.H.	10	F	Normal	22.4
J.P.	18	F	Normal	37.4
H.K.	35	M	Normal	37.4
C.C.	26	M	Malignant insulinoma	27.2
C.M.	7	F	Leukoencephalopathy	39.4
A.F.	26	F	Thyrotoxicosis	27.6
P.T.	9	F	Thyrotoxicosis	41.6
S.M.	3	M	Hurler's syndrome	28.4
C.M.	8	M	Hurler's syndrome	29.9
B.M.	6	F	Hurler's syndrome	23.8
L.B.	11	M	Galactosemia	29.8
J.C.	19	F	Mongolism and hypothyroidism	21.8
D.D.	1	F	Von Gierke's disease	31.1
H.S.	14	M	Adenocarcinoma thyroid	30.6
Mean				29.6 ± 1.2*

* Standard error.

Nutritional Biochemicals Corporation and was reprecipitated twice from water with 99 per cent ethanol before use. Glucose-1-phosphate as the dipotassium salt was obtained from Schwarz Bioresearch, Inc. and adenosine-5′-phosphate as the sodium salt from Sigma Chemical Company. Glucose-6-phosphate was purchased from Nutritional Biochemicals Corporation as the barium salt. Glucagon was obtained from Eli Lilly and Company.

RESULTS

I. Glycogen storage disease. The levels of leukocyte phosphorylase activity in the family studied are summarized in Table I. Both Jo.S.A. and R.A. have levels below 10 μg per 10^7 leukocytes. While the mother's level is midway between the lower limit of normal and that of the two patients, it was also lower than any of the control patients studied. The father and unaffected brother have normal levels of leukocyte phosphorylase. Despite these low levels of leukocyte phosphorylase in the affected members of the family, the glycogen content of the leukocytes was not elevated, as was liver glycogen. Sidbury, Cornblath, Fisher and House (20) have recently reported elevated erythrocyte glycogen in patients with debranching enzyme deficiency but normal levels in other types of glycogen storage disease. However, their series did not include patients with low hepatic phosphorylase.

II. Normal values. Leukocyte phosphorylase levels were determined in 20 patients and normal subjects, a total of 27 determinations being made (Table II). The mean level was 29.6 μg inorganic phosphorus liberated per 10^7 leukocytes with a standard error of $\pm$ 1.2 μg and a range of 20.6 to 41.6 μg. The youngest patient tested, a 1 year old female child (D.D.) with clinical Von Gierke's disease,[2] had a level of activity near the mean for the group; the values in the other children tested were indistinguishable from those found in adults. One of the control subjects, P.M., was studied on 4 different days and the level of leukocyte phosphorylase varied between 25.4 and 31.7 μg.

Wagner has reported that most of the glycogen in leukocytes resides in the polymorphonuclear types (13). The clear-cut difference between the patients with glycogen storage disease and the controls remained when the phosphorylase levels were corrected for percentage of polymorphonuclear leukocytes present in the fibrinogen supernatant. Since there was a 1 : 1 contamination of leukocytes with erythrocytes in the supernatant, phosphorylase activity of the erythrocyte was measured. No phosphorylase activity was detected in the erythrocytes, using approximately 1,000 times as many erythrocytes as the usual number of leukocytes in any one determination. Glucose-6-phosphatase activity was not demonstrable in leukocytes.

[2] Enzyme studies on the liver from this patient were not available at the time of writing.

TABLE III

In vitro effect of glucagon on leukocyte phosphorylase

Patient	Leukocyte phosphorylase	
	Control level	After glucagon
	μg $P/10^7$ WBC	
A.A.	3.7	11.5
Jo.S.A.	2.3	7.2
R.A.	0	1.4
Controls*	29.1 $\pm$ 3.9†	40.5 $\pm$ 6.5†

* Average of 5 separate determinations.
† Standard error; the mean stimulatory effect of glucagon is significant ($p < 0.05$).

In vitro addition of glucagon caused a 20 per cent ($p < 0.05$) stimulation of active phosphorylase in normal leukocytes. When glucagon was added *in vitro* to leukocytes from R.A., Jo.S.A. and A.A. there was a slight stimulation of phosphorylase activity in each, but in no case did the stimulated value approach the normal range (Table III). Adenosine-5′-monophosphate (0.02 M) had no significant effect on leukocyte phosphorylase activity.

When an aliquot of the sonicated leukocytes from R.A. was added to an equal volume of normal sonicated leukocytes, no inhibition of phosphorylase activity in the normal leukocytes was noted.

DISCUSSION

The demonstration of abnormally low levels of phosphorylase in the leukocytes of two patients with glycogen storage disease of the hepatic phosphorylase-deficient type indicates the usefulness of this type of study in establishing the diagnosis. The accessibility of these cells as contrasted with liver emphasized their advantage from a diagnostic point of view. Unfortunately it was not possible to study leukocyte phosphorylase in all the types of glycogen storage disease, although the level in a patient with the glucose-6-phosphatase-

deficient type was normal. It will be important to determine leukocyte phosphorylase levels in all the types before this test can be said to be entirely diagnostic of the hepatic phosphorylase-deficient type. Since there is no significant phosphorylase activity in erythrocytes, contamination of leukocytes with small numbers of erythrocytes does not alter the results. In these two patients there was good correlation between the levels of liver phosphorylase determined in biopsy specimens and the values found in leukocytes; i.e., liver phosphorylase in Jo.S.A. was estimated to be about one-third normal and in R.A., about one-sixth normal. Neither case had complete absence of phosphorylase and in this respect they are similar to the cases previously reported by Hers (11).

A relationship between liver and leukocyte phosphorylase is also apparent from the observation that in leukocytes from control subjects it was possible to activate phosphorylase by the *in vitro* addition of glucagon. This compound increases active phosphorylase in liver but has no effect on the muscle enzyme (21). The slight stimulation of leukocyte phosphorylase in the two patients and their mother does not permit a differentiation between a deficiency of the phosphorylase activating system and a decrease in the total amount of inactive phosphorylase present. Conversion of inactive liver phosphorylase to the active form is stimulated by epinephrine and glucagon through an adenyl cyclase system which increases the amount of adenosine-3′,5′-(cyclic) monophosphate (22). This in turn stimulates a specific kinase system requiring ATP and Mg^{++}, thereby converting inactive phosphorylase to the active form (23). Since leukocyte phosphorylase seems to respond to glucagon, this same system presumably should be operative in leukocytes, and low levels of the enzymes involved in this activating system might give decreased leukocyte phosphorylase activity, as would a low level of inactive phosphorylase. Nirenberg (24) has described a lack of both total and inactive phosphorylase in ascites tumor cells, where a normal activation system exists.

A defect in liver phosphorylase has only recently been described by Hers (11) in three patients with glycogen storage disease. In these patients (one male, two females) liver phosphorylase was found to be about one-seventh of normal, while muscle phosphorylase was normal, as was glucose-6-phosphatase and amylo-1,6-glucosidase. Hers was unable to stimulate active phosphorylase in the liver of these patients by addition of ATP and Mg^{++}, or adenosine-3′,5′-(cyclic) monophosphate. Another condition involving phosphorylase has been described by Mommaerts and co-workers (9) and Schmid, Robbins and Traut (10) in a syndrome represented by a "progressive myopathy" in which muscle phosphorylase was absent. Leukocyte phosphorylase has not been studied in patients with myopathy characterized by low muscle phosphorylase, but since leukocyte phosphorylase appears to mimic the liver enzyme, normal leukocyte phosphorylase levels would be expected in this disease.

The low level of leukocyte phosphorylase in these patients supports the theory that a specific phosphorylase-deficient type of glycogen storage disease exists as a distinct entity. The presence of a low leukocyte phosphorylase level in the mother suggests that the heterozygous state may be demonstrable in relatives of patients with this disease. However, the normal leukocyte phosphorylase in the father is against an autosomal recessive type of inheritance, such as has been suggested for other forms of glycogen storage disease (4). A sex-linked type of inheritance is unlikely in that the three patients presented by Hers (11) were both male and female. Further study of families with this disease may help settle this problem.

The recent studies of Leloir, Olavarria, Goldemberg and Carminatti (25) have shown a uridine diphosphoglucose-linked system for glycogen synthesis which is now thought to be the major synthetic pathway for glycogen in the body. The presence of normal leukocyte glycogen with low leukocyte phosphorylase in these two patients is thought to be further proof that the pathways of glycogen synthesis and breakdown are separate.

SUMMARY

Phosphorylase activity in leukocytes has been measured and found to be low in two cases of glycogen storage disease of the hepatic phosphorylase-deficient type. There was good correlation between the relative amounts of the enzyme in the liver and the leukocytes of these patients.

The mother had levels intermediate between the patients and control subjects but the remainder of the family members had normal levels. Since leukocyte phosphorylase seemed to vary with hepatic phosphorylase and since leukocytes are easily obtainable, it is suggested that this may be used as a diagnostic test for this condition. The low level of leukocyte phosphorylase in the mother of the two patients suggests that this test may be useful in determining the heterozygote state in this condition.

ACKNOWLEDGMENTS

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THE INCORPORATION OF PLASMA FREE FATTY ACIDS INTO PLASMA TRIGLYCERIDES IN MAN *

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Plasma triglycerides (neutral fat) may be divided into *1*) that portion which is present during the fasting state and *2*) that which, after ingestion of fat, is added to and temporarily augments the fasting level of triglycerides.

The present investigation deals with the relationship of plasma free fatty acids (plasma FFA) to fasting plasma triglycerides (plasma TG) in man. From these observations a quantitative assessment of the amount of plasma FFA converted to plasma TG will be presented. The data also permit calculations which suggest that plasma FFA may be the major source of fasting plasma TG.

METHODS

Two types of studies were carried out in male patients without known abnormalities of fat metabolism.

1. Non-reinjection experiments. After an overnight fast of 15 hours, 9 subjects were given 0.004 to 0.008 mc albumin-bound palmitic acid-1-C^{14} intravenously either by single injection or by 2-hour constant infusion. Two individuals received 0.00585 mc of albumin-bound oleic acid-1-C^{14} and another 0.00597 mc of stearic acid-1-C^{14}. During the day of the study the subjects were permitted no breakfast, a nonfat lunch, and an unrestricted supper. Venous blood samples were drawn at appropriate intervals over a period of 24 hours after administration of fatty acid C^{14} for determination of neutral fat activity. Two-ml aliquots of plasma were extracted by the method of Dole (1). Eight ml of the upper phase, representing 1.85 ml of plasma, was transferred to a separate tube. Ten ml of 0.1 N NaOH in 50 per cent ethanol was added, according to the method of Borgström (2), in order to remove fatty acids from the upper phase. The upper phase and its contents were then transferred quantitatively to liquid scintillator vials, dried with a stream of warm air from a hair dryer, and redissolved in 5 ml of phosphor solution for counting in a Packard Tri-Carb liquid scintillation spectrometer. Along with plasma glycerides, the extracts contained cholesterol, cholesterol esters and a small amount of phospholipid. These would also be expected to become labeled; therefore, it was necessary to determine the amount of activity in the various plasma lipids present in the extracts at various times after injection of C^{14}-labeled fatty acids. This was done by means of silicic acid chromatography using the method of Hirsch and Ahrens (3) without modification. Plasma palmitic acid-1-C^{14} activity was measured as previously described (4).

The experimental plan required a method which would permit easy and reproducible determinations on a large number of samples. The method selected for measuring plasma TG activity described in the preceding paragraph is based on the facts that: *1*) the label in the extracts appeared almost exclusively in TG in the early hours of the procedure, and *2*) negligible activity was present in cholesterol, cholesterol esters and phospholipid until at least 4 hours had elapsed. Furthermore, although significant activity appeared in phospholipids, only a small fraction of this phospholipid activity was present in the upper phase extracts, and activity in free cholesterol was minimal. The data justifying the method appear below.

Total plasma triglycerides were determined by the method of Van Handel and Zilversmit (5), plasma FFA by the method of Dole (1) and esters of carboxylic acids by the method of Rapport and Alonzo (6). Free cholesterol was precipitated as the digitonide and redissolved in methanol for liquid scintillation counting. Samples of expired air were analyzed for $C^{14}O_2$ activity by trapping in Hyamine hydroxide according to the method of Fredrickson and Ono (7).

2. Reinjection experiments. In order to establish with greater certainty the validity of using the rate of disappearance of TG label observed during non-reinjection experiments to measure fasting plasma TG turnover rate, a patient, terminally ill with degenerative cerebellar disease, was given 30 μc palmitic acid-1-C^{14} intravenously. Blood samples were drawn at intervals for determination of triglyceride activity. One hour after injection of the

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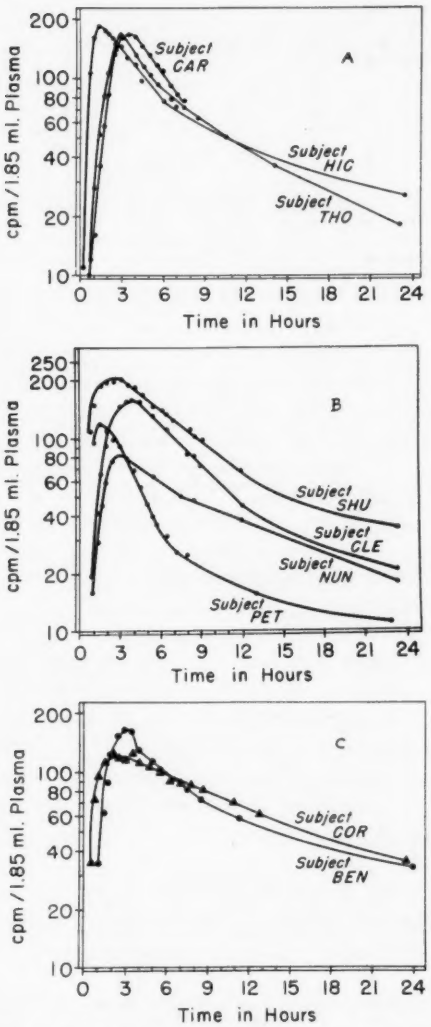

FIG. 1. PLASMA TRIGLYCERIDE ACTIVITY AFTER INTRAVENOUS INJECTION OF C^{14}-PALMITATE. Triglyceride activity represented by ascending limb and initial straight downslope only (see text).

radiopalmitate, 400 ml of blood was withdrawn and analyzed for TG and FFA activity. The blood was collected in a standard evacuated blood donor set containing acid citrate dextrose solution as anticoagulant and stored at 4° C. There is no evidence that this procedure does or does not denature or alter lipoproteins. A known quantity of plasma obtained from this blood was reinjected into another subject several days later.

This study was repeated using another subject who served both as donor and as recipient. Plasma neutral fat activity was determined in both donors and recipients as described in the preceding section.

RESULTS

I. Non-reinjection experiments

A. Triglyceride activity.[1] The results in nine normal persons are presented in graphs in which the logarithm of activity is plotted against time (Figure 1). After rapid intravenous administration of albumin-bound palmitic acid-1-C^{14}, there was a delay of about 10 to 15 minutes before activity began to appear. After the initial delay just described, activity rose rapidly and reached a peak in 2 to 4 hours, a time when FFA activity was very small. In the constant infusion experiments, a longer time was required to reach peak activity; otherwise these were not different from the single injection experiments (see Table I). (The purpose of the constant infusion studies was to simplify the kinetic problem by attempting to produce a situation in which the specific activity of TG influx into plasma was constant. This was later found to be both unnecessary and impractical.) Thereafter activity declined relatively rapidly at first and then more slowly over the 24 hour period of observation. Initially there was a straight line exponential decline after attainment of peak plasma activity. There was some uncertainty about this in two studies (Figure 1C). In all cases there was a departure from linearity beginning, at the earliest, 6 hours after injection of palmitic acid-1-C^{14}. Figure 2 shows activity in TG 1 and 2 hours after injection of FFA. At 1

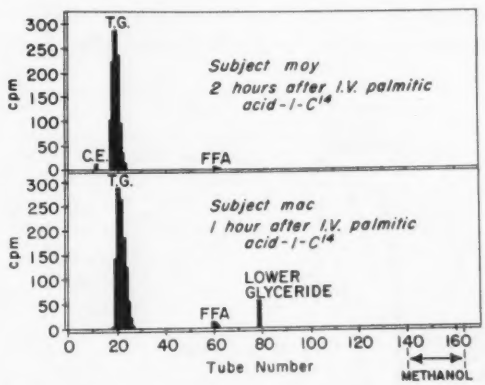

FIG. 2. SILICIC ACID CHROMATOGRAM OF PLASMA, 1 AND 2 HOURS AFTER INJECTION OF C^{14}-PALMITATE BY GRADIENT ELUTION METHOD.

[1] The curves described are a measure of total activity in plasma extracts.

TABLE I

*Observed and calculated plasma triglyceride fatty acid contents and data used to obtain calculated levels in 9 subjects given palmitic acid-1-C^{14} **

	1	2	3	4	5	6	7	8	9	10	11	12	13
Subject	Plasma TGFA content	Calculated plasma TGFA content	Deviation	x Plasma TG activity at time t_1	y At time t_1	s At time t_1	Average FFA conc.	FFA flux	K Fractional disappearance rate of plasma TG per hr	F Fraction of FA label appearing in plasma	aF Calculated plasma TG influx	FFA administered	Calculated plasma volume
	mmoles/total plasma vol		%	*cpm/1.85 ml plasma*			*mmoles/L*	*mmoles/hr*			*mmoles/hr*	*cpm*	*ml*
CAR†	11.8	5.9	−50	145	93.0	238.0	670	27.6	0.217	0.046	0.425	6.7×10^6	2,290
THO†	6.5	5.9	− 9	94	118.5	212.5	700	29.0	0.232	0.047	0.458	5.86×10^6	2,300
BEN†	12.0	9.8	−19	70	110.5	180.5	450	26.1	0.123	0.046	0.400	6.12×10^6	2,900
NUN†	30.1	20.5	−35	45	63.5	108.5	600	50.3	0.115	0.059	0.783	5.51×10^6	4,670
CLE†	15.3	24.0	+50	130	91.9	221.9	700	34.3	0.165	0.093	1.067	4.84×10^6	3,270
HIC‡	9.9	10.4	+ 4.5	77	138.6	215.6	650	30.3	0.201	0.069	0.695	4.62×10^6	2,950
SHU‡	15.5	13.1	−14	90	164.0	254.0	500	26.2	0.124	0.069	0.542	6.78×10^6	2,910
COR‡	17.0	18.6	+ 9	106	49.2	155.2	330	26.9	0.078	0.078	0.627	4.32×10^6	3,400
PET‡	4.7	5.0	+ 7	39	104.5	143.5	1,000	46.6	0.347	0.042	0.646	5.13×10^6	2,330

* For explanation of x, y and s, see text. Columns 1 and 2 give measured and calculated triglyceride concentrations; columns 4–13 give data used to obtain calculated levels in column 2 by methods described in the text.
† Constant infusion studies.
‡ Single injection studies.

hour no activity was detected in cholesterol esters, and at 2 hours 1.5 per cent of the total activity was found in this fraction.

B. CO_2 activity. Figure 3 illustrates the appearance of CO_2 activity in expired air in relation to palmitic acid and TG activity during a constant infusion experiment and also illustrates the fact that peak expired CO_2 labeling occurs before plasma TG peak activity.

C. Phospholipids. Table II shows the amount of activity in phospholipids at various intervals after the beginning of the studies. These determinations were performed on separate samples of plasma by silicic acid chromatography using methanol: chloroform (1 : 2, vol/vol) for extraction of plasma.

D. Cholesterol esters. Figure 4, a representative example performed as a separate study, shows the appearance of label in cholesterol esters. Activity is initially small, but in 24 hours is responsible for a significant portion of the activity of the extracts. This can be ascertained by comparing the amount of cholesterol ester activity in Figure 4 with the residual total activity in Figure 1, A and B.

E. Free cholesterol. Activity in free cholesterol constituted only a fraction of background activity.

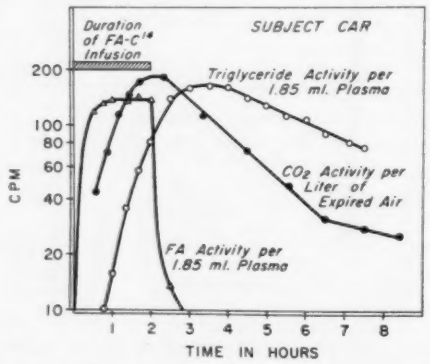

FIG. 3. APPEARANCE OF CO_2 ACTIVITY IN EXPIRED AIR IN RELATION TO PLASMA FREE FATTY ACID AND TRIGLYCERIDE ACTIVITY.

TABLE II

Appearance of phospholipid activity in plasma after injection of palmitic acid-1-C^{14}

Subject	Time after palmitic acid-1-C^{14} administration	Radioactivity
	hrs	*cpm per 1.85 ml plasma*
CAR	1	20.4
	2.5	39
	5.5	41
THO	1	19
	3	20
	5.5	26
	22.5	40
CLE	1	5
	3	17
	5.5	60

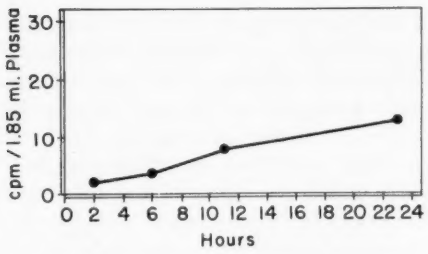

FIG. 4. APPEARANCE OF CHOLESTEROL ESTER ACTIVITY AFTER INTRAVENOUS PALMITIC ACID-1-C^{14}.

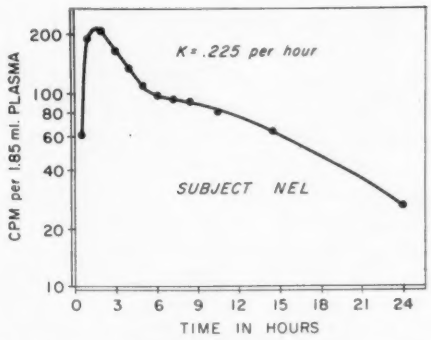

FIG. 5. PLASMA TRIGLYCERIDE ACTIVITY AFTER INJECTION OF C^{14}-OLEATE. Triglyceride activity represented by ascending limb and initial straight downslope only (see text).

F. Oleic acid. The early part of the curves obtained (Figure 5 is a representative example) resembled the curves seen in the palmitic acid studies. The fractional turnover rates for the early TG portions of the curves were 0.225 and 0.314 per hour, respectively. Later the form of the curve was different. Duplicate determinations on separate samples of plasma revealed identical results. It is also quite clear from inspection that the amount of oleate label converted to TGFA label and the rate of disappearance are in the same range as these values for palmitate. Silicic acid chromatography of plasma obtained at 9 hours revealed that the difference in appearance between this curve and the palmitic acid curve is accounted for by a greater amount of activity in cholesterol esters. This is consistent with the known fact that cholesterol esters contain greater amounts of unsaturated acids (8).

G. Stearic acid. The form of the stearic acid curve resembled those for palmitic acid. The decline of radioactivity after attainment of peak activity was a straight line on the semilogarithmic plot and the fractional turnover rate was 0.146 per hour, a value which fell within the range of values obtained for palmitic acid. The curve was different in that maximal activity was only 51 cpm per 1.85 ml plasma, a value which was considerably lower than any obtained for palmitic acid (see Figure 1).

II. Reinjection experiments

The results of the two reinjection studies are summarized in Table III. In the first study, results indicated that the volume of distribution of the reinjected triglycerides was 25 per cent greater than the calculated plasma volume based on 40 ml plasma per kg body weight. The rate of disappearance of TG activity in the recipient was very

TABLE III

Plasma triglyceride fractional disappearance rates in reinjection studies and related data

	K Fractional disappearance rate of plasma TG per hour	No. of cpm injected	Estimated plasma volume*	Expected activity at zero time	Actual extrapolated activity at zero time
Expt. 1:					
Donor	0.422				
Recipient	0.402	26,300	3,000	43.8 cpm per 5 ml plasma	35 cpm per 5 ml plasma
Expt. 2:					
Donor					
Recipient	0.282	17,050	2,200	77.5 cpm per 10 ml plasma	59 cpm per 10 ml plasma

* Estimated plasma volume based on 40 ml per kg body weight.

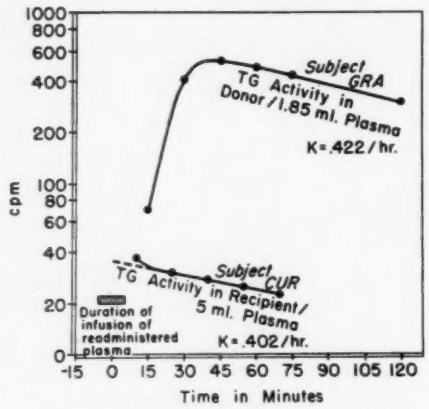

FIG. 6. PLASMA TRIGLYCERIDE ACTIVITY IN REINJECTION STUDY.

close to the initial rate of TG disappearance after attainment of peak activity in the donor (Figure 6), but in both donor and recipient was more rapid than in the non-reinjection studies. When the experiment was repeated using the same individual as donor and recipient, only the rate of decay of the reinjected material could be observed, and confirmed the previous findings that the volume of distribution corresponds rather closely (25 per cent greater) to the plasma volume and that the rate of disappearance was of the same order of magnitude observed in the non-reinjection experiments. Uptake of labeled TG during the period of infusion and possible equilibration with tissue TG are factors which may have been responsible for an overestimation of the size of the TG pool. In our first reinjection experiment the fractional disappearance rate was 0.402 per hour, a rate which was close to but higher than those found in our non-reinjection studies. However, in the second reinjection experiment, the fractional disappearance rate was 0.282 per hour, a value which fell within the range of the non-reinjection studies.

INTERPRETATION

I. Background

The studies in man of Fredrickson and Gordon (9) demonstrated that a portion of intravenously administered C^{14}-labeled fatty acids is later found in plasma as labeled neutral fat. There is good evidence that this conversion is effected in liver. Thus Stein and Shapiro (10), and Laurell (11) found that this organ removed intravenously administered radiopalmitate and that activity was found in liver triglycerides and then in plasma triglycerides. In the experiments of Laurell, the specific activity of liver and plasma triglycerides appeared to be the same so that these findings were thought to be compatible with the concept that fasting plasma triglycerides might be entirely derived from liver triglycerides. On the other hand, Stein and Shapiro believed that liver triglycerides mix with a larger triglyceride pool from depots (10).

II. Discussion of results

A. Reinjection experiments. The observations in man made in this study differ from those in the rat in at least two obvious particulars. First, the rate of disappearance in the non-reinjection studies of Laurell is more rapid than that in man. Second, the rate of disappearance in the rat of reinjected fasting triglycerides is much more rapid than in the non-reinjection experiments (11). In our own studies this difference was either not observed or remained in doubt (see Figure 6 and compare K values in Tables II and III). However, unpublished observations in this laboratory in dogs resemble those made in the rat by Laurell. From our own reinjection studies we conclude that the rate of disappearance resembled a simple first order decay and that the volume of distribution of the reinjected TG approximated the calculated plasma volume.

B. Non-reinjection experiments. The activity in an extract containing triglycerides, cholesterol esters, free cholesterol and some phospholipids is considered, on the basis of silic acid chromatographic studies, to represent triglyceride activity alone during the ascending portion of the curve and during the initial descending portion of the curve which forms a straight line on the semilogarithmic plot. Furthermore, the straight logarithmic downslope is considered to be representative of the disappearance of a single substance. This would be valid except in the unusual circumstance in which two or more different substances happened to be disappearing at the same fractional rate. The departure from linearity which occurs several hours after injection of labeled fatty acid

can be explained either by recirculation of labeled triglyceride or by the appearance of another labeled material. The results, in fact, demonstrate the late appearance of activity augmenting the residual TG activity, both in cholesterol esters and in phospholipids.

III. Current hypothesis

The purpose of the studies on the relationship of C^{14}-labeled fatty acids to plasma C^{14}-labeled triglycerides was to evaluate the quantitative significance of this conversion in man.

As stated previously, it is believed, from the data of Laurell (11), Stein and Shapiro (10), and Hillyard, Cornelius and Chaikoff (12), that fatty acid is converted to triglyceride in the liver and that plasma TG may be in equilibrium with liver TG (11). However, in the present investigation, since only plasma levels were measured, it will be necessary to limit the interpretation arbitrarily to events occurring in the plasma pool even though this pool might be only a fraction of a larger TG pool with which TG equilibrates rapidly. The fractional rate of disappearance of TG-C^{14} calculated from the TG activity curves is interpreted to represent the rate of disappearance of fasting plasma triglycerides in general. It should be stated in this regard that TG molecules in blood are composed of a mixture of fatty acids (13) and are believed to be removed intact by adipose tissue *in vitro* (14). Another point in support of the hypothesis that TG is removed intact is the observation in this study that both oleic and palmitic acids, the two most abundant compounds in this class, behaved in the same way and fell into the same range of disappearance rates from plasma. It is also known that lower glycerides do not normally appear in plasma (3). Since TG molecules are composed of a mixture of fatty acids, one would anticipate that lower glycerides would be present in plasma if the rate of disappearance were more rapid for one species of fatty acid than for another. Futhermore, it is assumed that the calculated fractional rate is also the rate of disappearance for TG throughout the course of the study, especially during the time of increasing triglyceride activity.

Another important assumption, relating to the method of interpretation which follows, involves the concept that once peak plasma triglyceride activity has been reached and the straight exponential downward decay begins, no further *net* addition of TG label into the TG pool takes place. The word *"net"* is emphasized since a straight line could be observed if plasma TG equilibrates rapidly with other TG pools, i.e., if plasma TG were part of a larger TG pool from which TG disappearance proceeded at a uniform rate. The question that this investigation attempts to answer does not concern the total magnitude of conversion of FFA to TGFA, but whether enough FFA becomes TGFA to account for the observed TG concentration in plasma. The concept of "no further net addition" is justified by the following arguments. First, further net entry of label would alter linearity. Second, a model which fits the observed data was based on the observed rate of TG decay and a rate of entry into plasma also approximated from the data. The following equation was derived:

$$x = \frac{k_1 z_0}{k_2 - k_1} (e^{-k_1 t} - e^{-k_2 t})$$

where x is plasma TG activity, k_1 is the rate of disappearance from the pool emptying into plasma, k_2 is the plasma TG fractional disappearance rate, and z_0 is the amount of TG label released or equilibrating into plasma (15). Plotting this equation with values approximating those obtained experimentally gives a curve resembling the experimental curves. This equation, furthermore, is based on the assumption that release of TG label into plasma is exponential with time, an assumption which is consistent with the form of the increasing limb of the triglyceride activity curves. Third, inspection of the curves indicates that label equilibrates very rapidly at first and that this rapid rate of entry slows promptly and, therefore, the net influx of label would be expected to become rapidly insignificant and approach zero. The consequence of this would be that only the rate of disappearance of label would be manifest and that this picture would be uncomplicated by further addition of labeled material.

In addition to the foregoing, two other facts are pertinent to the discussion. First, plasma FFA is composed of about 50 per cent oleic acid, 30 per cent palmitic acid, 8 per cent stearic acid and several per cent of various other fatty acids

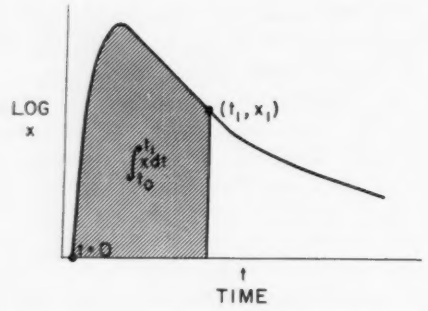

FIG. 7. DIAGRAMMATIC REPRESENTATION OF DATA ILLUSTRATING METHOD OF CALCULATION. Explanation in text.

(16). Second, the fatty acid composition of triglycerides in plasma is very similar—approximately 40 per cent oleic, 30 per cent palmitic, 10 per cent linoleic, 5 per cent stearic, 5 per cent palmitoleic and varying smaller amounts of other fatty acids (8).

The presentation which follows is not intended to represent an accurate description of the kinetics of the individual studies but serves to indicate in a general way the magnitude of the conversion of FFA to plasma TG and to suggest that plasma FFA are the major source of plasma TG. The fractional disappearance rate and volume of distribution of plasma FFA, the plasma volume, and the values derived from these quantities were not experimentally determined but are representative of known normal values and may be the source of considerable error in individual studies. The interpretation is presented below.

In order to take into account the likely situation that plasma TG may be in part of a larger TG pool probably including liver TG, it is necessary to interpret the data in a manner which is independent of pool size. The following model is pictured. FFA label is converted to TGFA label which then equilibrates rapidly with all parts of the TG pool including plasma. This pool may be the same size or larger than the plasma pool. Disappearance then proceeds at a uniform rate from the entire pool.

In Figure 7, the logarithm of plasma TG activity, x, is plotted against time, t. Let z = total TGFA activity (TGFA*) which has equilibrated with the plasma compartment, i.e., the total amount of administered fatty acid label (FA*), which appeared in plasma as TGFA*. Let y = amount of TGFA* which has left plasma from time $t = 0$ to $t = t_1$; t_1 is any point in time on the straight downlimb of the semilogarithmic plot. Let M = amount of FA label administered. Let F = fraction of FA label converted to plasma TGFA*. F equals z/M. Let a = endogenous FFA turnover rate or flux. Let k = fractional disappearance rate of plasma TG. Then at any time t, on the straight downlimb of the semilogarithmic plot, $z = x - y$; dy, the amount of TGFA* which has left plasma during an interval of time, dt, $= kx\,dt$, and the total amount of TFGA*, y, which has left the

plasma from $t = 0$ to $t = t_1 = \int_{t_0}^{t_1} kx\,dt$

and $z = x - k\int_{t_0}^{t_1} x\,dt$.

But $F = z/M = \left[x - k\int_{t_0}^{t_1} x\,dt\right]/M$.

The amount of TGFA formed per hour which enters plasma equals the fraction of FA label converted to plasma TGFA* times the endogenous FFA turnover rate. This equals Fa, which equals $a/M\left[x - k\int_{t_0}^{t_1} x\,dt\right]$.

If TGFA enters plasma at the rate of Fa millimoles per hour and leaves at the fractional disappearance rate k, the anticipated plasma TGFA content which will be reached can be expressed by $Fa/k\ (1 - e^{-kt})$ (17).

When $t = \infty$, plasma TFGA content will equal Fa/k; or, substituting $Fa = a/M\left[x - k \times \int_{t_0}^{t_1} x\,dt\right]$, plasma TGFA content $= a/Mk \times \left[x - k\int_{t_0}^{t_1} x\,dt\right] = a/M\left[x/k - \int_{t_0}^{t_1} x\,dt\right]$. The integral $\int_{t_0}^{t_1} x\,dt$ is the area under the shaded portion of the curve. This area was determined in these studies by the trapezoid rule. The area depicted in the diagram is, of course, not correct since it is drawn under semilogarithmic plot. The correct area would be represented by the equivalent area drawn under an arithmetic plot. In practice the semilogarithmic diagram was used to facilitate the

measurement of k and to insure that x_1 is measured on the straight semilogarithmic downslope.

Note that when $t_1 = \infty$, $x = 0$ and plasma TGFA $= a/M \int_{t_0}^{t_1} x\,dt$. The expression $1/M \times \int_{t_0}^{t_1} x\,dt$ equals the area under the TGFA curve from $t = 0$ to $t = \infty$ divided by the total amount of FA label administered.[2]

In calculating the data with the equations presented, the following factors were taken into account: *1*) Plasma volumes were calculated on the basis of 40 ml plasma per kg body weight (18). *2*) It was assumed that the fraction of palmitic acid-1-C^{14} converted to TGFA* is representative of fatty acids as a whole. Whereas this is not strictly true it is a very reasonable approximation, since the fatty acid composition of plasma FFA and plasma TGFA is almost identical (8, 16) and since oleic and palmitic acids behaved similarly. *3*) Endogenous hourly FFA turnover rate, a, was estimated from the average plasma FFA level during the period of TGFA* formation by using a fractional disappearance rate of 0.3 per minute (9). The plasma volume was taken as the volume of distribution of FFA, although the latter volume may be somewhat greater than the former (4, 9). *4*) Extraction efficiency of 87 per cent for FFA and 95 per cent for TG was taken into account.

The data and method of calculations outlined above were used to estimate an anticipated plasma triglyceride content for each subject based on a rate of TG formation calculated from the isotope data. The calculated levels would represent a minimal estimate if recirculation of TGFA occurs. These anticipated or theoretically expected levels derived from the tracer data were compared with the actual TGFA plasma contents in each subject. The actual TG contents with which the calculated levels were compared were the means of several levels obtained during the period of formation of labeled TGFA. During this time there was little fluctuation in TG concentration. The data and calculations appear in Table I. Plasma TG content was calculated from the determinations by using a mol wt of 900. It can be seen (columns 1, 2 and 3) that the calculated approximations range from 50 per cent less to 50 per cent more than the measured levels and that six fall within 19 per cent of the measured levels. The same results can be obtained in a somewhat different manner: the plasma TGFA efflux is the product of k and TGFA content and this efflux can be compared with aF, the calculated rate of entry of plasma TGFA. Under these circumstances the results are unaltered. Since calculated TGFA flux into plasma approximates endogenous TGFA flux out of plasma, it would appear from these calculations that all three fatty acids of triglyceride turn over and that the majority of plasma TG originated from FFA. That the major fatty acids of TG turn over at the same rate is strongly suggested not only from a comparison of the results with palmitic acid-1-C^{14}

[2] Since the writing of this paper an alternative method of analyzing the data has been proposed. The derivation which follows is more general in nature, and under certain circumstances requires no knowledge concerning the rate of disappearance of TGFA.

Let ω = time of introduction of labeled FA. Let M = total dose of labeled FA. Let $f(t - \omega)$ = fraction of a given quantity of FFA which enters plasma at time ω and which is to be found with plasma TGFA pool at time t (a steady state is assumed). Let $m(\omega)$ = plasma FFA flux (rate of entry of FFA into plasma). Then, the quantity of FFA which entered plasma during time interval $d\omega$ is $m(\omega)d\omega$.

At time t from the time this quantity, $m(\omega)d\omega$, entered, the quantity $f(t - \omega)m(\omega)d\omega$ would be found in the plasma TGFA pool, or adding up for all previous time intervals we obtain:

$$\text{total plasma TGFA} = \int_{-\infty}^{t} f(t - \omega)m(\omega)d\omega$$

The substitution $\Omega = t - \omega$ transforms this into the equivalent expression:

$$\text{total plasma TGFA} = \int_{0}^{\infty} m(t - \Omega)\, f(\Omega)d\Omega$$

If the rate of entry of FFA into the plasma is constant, say $m(\omega) = a$, then the quantity of TGFA present at time t will be:

$$\text{total TGFA} = a \int_{0}^{\infty} f(\Omega)d\Omega$$

The integral $\int_{0}^{\infty} f(\Omega)d\Omega$ is the total area under the plasma TGFA curve divided by the total initial dose of labeled fatty acid. It can be seen immediately that the equation TGFA $= a \int_{0}^{\infty} f(\Omega)d\Omega$ is identical with the equation presented in the main text when $t = \infty$.

The alternative equation for practical application requires extrapolation of the straight semilogarithmic downlimb of the TGFA* curves to infinity in order to measure the required area.

and oleic acid-1-C^{14}, but also from data of Rodbell showing that TG is removed intact by adipose tissue *in vitro* (14).

A point which should be emphasized is that the results, suggesting that plasma FFA conversion to plasma TGFA is sufficient to account for plasma TG concentration, would be the same if these calculations were based on a unit volume of plasma; i.e., the interpretation is independent of pool size. Furthermore, the value obtained for the fraction of radiopalmitate converted to plasma TGFA applies only to the initial equilibration of label into plasma. The actual fraction of FFA converted to TGFA might be considerably in excess of this amount if one considers, as Laurell claims (11), that plasma TG specific activity might well be the same as that in liver and that liver TG probably greatly exceeds plasma TG.

DISCUSSION AND CONCLUSIONS

An important concomitant of the present interpretation is the concept that one of the factors relating directly to the plasma TG level is the magnitude of plasma FFA turnover rate. This idea finds support in observations relating plasma FFA concentration to plasma TG concentration. For example, Havel (19) found that administration of glucose, which causes a fall in plasma FFA, also causes a fall in plasma TG concentration. Dury and Treadwell (20) showed that epinephrine which raises plasma FFA concentration also raises plasma TG concentration. Both diabetes and starvation produce a rise in plasma FFA and plasma TG.

Another point which should be made is that the present study does not distinguish actual triglyceride formation from a process which exchanges one TGFA for another from FFA. There is, however, suggestive evidence from the studies of others that FFA may not be released from the liver. The large positive FFA arteriovenous difference of the blood perfusing liver (21) suggests that such an exchange may not be the only mechanism concerned. Stein and Shapiro were unable to find significant amounts of FFA label after uptake of C^{14}-triglycerides by rat liver (22). This last point is, however, difficult to evaluate because liver FFA is rapidly diluted by a large FFA flux from plasma. It has also been shown that plasma triglycerides are removed by adipose tissue *in vitro* (14, 23), and that only a fraction of administered β-lipoprotein triglyceride is taken up by liver (22). If plasma triglyceride undergoes a fate other than recirculation to liver and if, as has been shown in these isotopic studies, the majority, and perhaps all plasma TG originates from liver, it would be unlikely that incorporation of FFA into TGFA would be entirely an exchange phenomenon. However, proof of this contention is not possible at present.

It is also necessary to point out evidence concerning the importance of the liver in the incorporation of FFA into TGFA and the lack of participation of extrahepatic tissues in contributing to the fasting triglyceride via an FFA precursor. Such evidence is forthcoming not only from animal studies already mentioned but also from the recent observation by Havel and Goldfien (24) that hepatectomy in dogs abolished the conversion of FFA to TGFA.

It is concluded from the data presented and from the interpretation of these data that plasma FFA is a major source of fasting plasma triglyceride. The calculations, furthermore, strongly suggest that FFA is involved in a process in which all three fatty acids of triglyceride turn over and that FFA is therefore the preponderant source of fasting TGFA.

SUMMARY

Observations have been made on the incorporation of injected C^{14}-labeled fatty acids into triglycerides, cholesterol esters, free cholesterol and phospholipids.

An estimate was made of the quantitative aspect of the formation of triglyceride label. The results of this estimate are consistent with the hypothesis that a major fraction of fasting plasma triglyceride is derived from plasma fatty acids.

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THE EFFECT OF METHEMOGLOBIN FORMATION IN SICKLE CELL DISEASE *

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On a biochemical level, sickle cell disease is perhaps the most completely understood disease of man. In spite of this, no satisfactory treatment is available to patients afflicted with this disorder.

The clinical manifestations of sickle cell anemia are varied. Some of the complaints of patients with this disease can be ascribed directly to the lack of oxygen-carrying capacity of the blood. Other symptoms, however, such as the painful crises, the leg ulcers and bone infarcts, are not due to anemia alone. They do not occur in other anemias of equal severity and must thus be ascribed to additional factors. One such factor might be the distortion which sickle cells undergo when the oxygen tension is lowered. Any means by which the shape change of red cells could be inhibited might, therefore, benefit patients with this disorder. Reports of attempts to modify the sickling process by changing the pH of the environment of red cells through the administration of sodium bicarbonate (1) or by poisoning red cell carbonic anhydrase with acetazolamide (Diamox) have been published (2). Clinical results of acetazolamide treatment have been disappointing (3). We are not aware of published confirmation of the effect of alkali therapy on the clinical course of sickle crises, although a recent study (4) showed that it was not possible to influence *in vivo* sickling by intravenous administration of bicarbonate. The effect of older therapies on sickle cell disease has recently been reviewed by Dacie (5). None is considered effective. To our knowledge, studies of the effect of any drug therapy on *in vivo* red cell survival have not been published.

A recent review (6) stimulated us to approach this problem from a different point of view. Itano (7) had demonstrated that complete conversion of sickle hemoglobin to the methemoglobin derivative prevented the sickling process. It seemed probable that conversion of only a part of the hemoglobin of sickle cells to methemoglobin would result in partial inhibition of the sickling process. The studies of Greenberg, Kass and Castle (8) have demonstrated that the mean corpuscular sickle hemoglobin concentration (MCSHC) was important in determining whether pronounced clinical symptoms of sickle cell disease were present. Those patients whose MCSHC values were less than 15 g per cent were not anemic and had no painful crises. Hemoglobin converted to methemoglobin would be removed from the oxyhemoglobin-reduced hemoglobin equilibrium. In this way the MCSHC of sickle cells would in effect be diminished when methemoglobin was formed. Thus, inhibition of sickling might take place in patients treated with methemoglobin-forming agents.

Furthermore, it has been shown that the lowest gelling point of mixtures of S hemoglobin with other hemoglobins depends not only on the absolute concentration of sickle hemoglobin in the solution, but also on the type of the other hemoglobins (9). In addition, sickling of red blood cells of infants with sickle cell trait has been found to correlate not with the absolute quantity of sickle hemoglobin in the red cells, but rather with the reciprocal of the amount of fetal hemoglobin present (10). Therefore, it appeared at least theoretically possible that methemoglobin might interfere with the sickling process to a greater extent than would be expected from its effect in removing hemoglobin from the $Hb \rightleftarrows HbO_2$ equilibrium, alone.

Proceeding on the premise that it may be possible to benefit patients with sickle cell anemia by inhibiting the sickling process, even if they are at

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TABLE I

Laboratory data on five patients with sickle cell anemia *

Patient	Hb	Hct	RBC	Retic.	Appearance of RBC on smear	Alkali-resistant Hb	Bilirubin	
							Direct	Total
	g %	*%*	*10^6/mm³*	*% RBC*		*% total*	*mg %*	
L.A.	7.3	27.5	2.80	11.5	Sickling, Howell-Jolly bodies, normoblasts, target cells			
B.W.	10.0	27	3.24	6.1	Sickling, many target cells	4.52	0.30	0.60
T.P.	7.37	23.5	2.65	14.3	Sickling	4.64		
J.G.	6.2	19	1.97		Sickling	3.90	2.22	3.34
J.R.	8.0	24	2.53	10.0	Sickling	3.44	0.80	3.80

* All 5 patients had SS hemoglobin shown by paper electrophoresis, and the glutathione stability test was normal in each.

the same time deprived of a certain quantity of oxygen-carrying capacity, we have undertaken an investigation of the effect of such chemical modification of sickle hemoglobin in patients with sickle cell disease. After these studies had been undertaken and a preliminary communication had been published (11), we learned that in his thesis Itano (7) had suggested that clinical investigations of the treatment of sickle cell disease with sodium nitrite might be carried out. At his suggestion, preliminary trials were carried out by Dr. J. C. S. Paterson. In long-term experiments, 300 to 500 mg sodium nitrite in divided daily doses was well tolerated and, although subjective improvement was noted, there was little evidence of hematological improvement. In acute experiments the side effects of sodium nitrite administration by infusion in anemic patients were found to be sufficiently troublesome to necessitate interrupting his studies. These observations have not been published (12).

MATERIALS AND METHODS

A. Patients. Five Negro women between the ages of 21 and 33 volunteered for these studies. The pertinent admission laboratory data are given in Table I. No blood transfusions were given to any of the patients during the course of the studies.

B. Determination of oxygen tension sickling curves. The following gas mixtures were obtained from a commercial source and the exact gas content assayed in each case [1] (except for the 1 per cent O_2 mixture): 0, 1, 3, 5, 10 and 20 per cent O_2. The CO_2 content varied from 8.1 to 10.0 per cent. The balance of gas was N_2. The actual O_2 content has been used in calculating the pO_2 throughout, except in the case of 1 per cent O_2. Each gas mixture was passed through a gas-washing bottle filled with distilled water.

It was found in preliminary experiments that consistently satisfactory results relating oxygen tension to percentage sickling could not be obtained on washed, resuspended red cells, so that it was necessary to carry out studies on whole blood samples. Accordingly, heparinized blood was obtained from each subject and 1 ml was introduced into a 500 ml separatory funnel (total volume, 650 ml) which was employed as a tonometer. In studies done on Subject T.P., 2.5 ml was used. The space in the separatory funnel was flushed with the appropriate gas mixture at a rate of 6 L per minute; it was stoppered and the stopcock closed. The funnel was then rotated at room temperature (22 to 25° C) in such a manner as to spread the blood in a thin film on the surface of the glass. After 5 minutes, the gas inside the funnel was again flushed out for 30 seconds at a flow rate of approximately 6 L per minute and the rotating process repeated. After a third flushing and period of rotation, the funnel was flushed with gas a fourth time for 30 seconds and a drop of blood was introduced under oil from the stem of the funnel into a fixative containing 5.0 per cent formalin,[2] 0.010 M Na_2HPO_4 and 0.138 M NaCl which had been saturated with the gas with which the blood sample had been equilibrated. This fixative was designed to give a final pH of from 6.6 to

[1] Courtesy of Mr. John Barr, chief technician, Cardiac Physiology Laboratory, City of Hope Medical Center.

[2] Solution of formaldehyde, 37 per cent by weight, USP, Merck.

6.8. After 15 to 30 minutes' fixation, a droplet of blood was introduced into a counting chamber and approximately 200 cells were enumerated; the percentage of distorted cells was recorded. The blood of patients without sickle hemoglobin but with a similar degree of anemia was studied to determine the control level of red cell distortion.

In most studies a red cell which deviated in any appreciable manner from a regular discoid shape was considered to be distorted. In one study more rigid criteria for sickling were employed: cells were considered to be distorted significantly only if the longest diameter of the cell was 2.5 times the shorter diameter, or if there were two distinct points on the cell.

C. Methemoglobin, hemoglobin, alkali-resistant hemoglobin and Heinz bodies. Methemoglobin and hemoglobin determinations were carried out by the method of Evelyn and Malloy (13), modified by the addition of 0.02 per cent saponin to the 0.0167 M buffer, and standardized against a commercially available cyanmethemoglobin standard (Acuglobin, Ortho Pharmaceutical Corp.). In measuring the hemoglobin and methemoglobin content of the fractional hemolysates produced by differential osmotic hemolysis, the method of Evelyn and Malloy was modified in that the determinations were carried out at a pH of 7.4 instead of 6.6. Appropriate conversion factors derived empirically for this pH were used.

The method of staining Heinz bodies has been described previously (14). Alkali-resistant hemoglobin was measured by a slight modification of the method of Betke, Marti and Schlicht (15): the erythrocytes were washed only once before lysis. Results were identical with those obtained when the original procedure was employed. Normally, less than 0.8 per cent of the hemoglobin is alkali-resistant when measured by this technique.

D. Drugs. Sodium nitrite was administered in the form of 1 grain tablets (Eli Lilly & Co.). Para-aminopropriophenone (PAPP) (Eastman Organic Chemical Corp.) was dissolved in propylene glycol at a concentration of 10 mg per ml. PAPP solutions were prepared at least weekly. Both drugs were administered by the oral route. The pharmacology of methemoglobin formation by sodium nitrite and PAPP has been studied in experimental animals and in human subjects and will be reported elsewhere (16). A single oral dose of sodium nitrite tablets or of PAPP dissolved in propylene glycol induces a peak level of methemoglobinemia within approximately 1 hour, with a gradually declining level thereafter. If either of these drugs is administered every 4 hours at the same dosage level, a gradual increase in the blood methemoglobin concentration occurs and the level of methemoglobin is exceedingly stable from hour to hour.

The chorionic gonadotropin administered to T.P. was obtained from Parke, Davis as Antuitrin S. J.R. received chorionic gonadotropin from Organon as Pregnyl and from Squibb as Follutein.[3] J.R. also received 10 mg of Premarin (Ayerst) and 200 mg progesterone (Pfizer) daily for a period of 10 days.

E. Oxygen dissociation curves. Oxygen dissociation curves were determined on aliquots of blood equilibrated in a separatory funnel as indicated above, collected under oil, and introduced into the Van Slyke apparatus (17), after hemoglobin and methemoglobin determinations had been carried out.

F. Serial osmotic hemolysis. Nine days after the intravenous administration of 10 μc of Fe^{59}-citrate, heparinized blood was drawn from Subject B.W. in order to carry out serial osmotic hemolysis studies. Ten ml of blood was centrifuged at 375 G for 10 minutes. The plasma was discarded and ice-cold 0.9 per cent NaCl was added to give a total volume of 30 ml. The suspension was centrifuged at 2° C at 170 G for 15 minutes and the supernatant discarded. At this temperature (and in the absence of glucose) no significant reduction of methemoglobin takes place. Buffered saline, prepared by the method of Dacie (18), equivalent to 0.65 per cent sodium chloride, was added to a total volume of 30 ml and the resuspended red cells were incubated at 0° C for 10 minutes. The suspension was then centrifuged at 2° C for 15 minutes at 170 G. The supernatant was removed for hemoglobin, methemoglobin, and radioactivity determinations. Buffered saline, equivalent to 0.60 per cent sodium chloride, was then added and the procedure repeated. Subsequently, buffered saline, equivalent to 0.55, 0.50, 0.45, 0.40, 0.35, 0.30, 0.25 0.20, and 0.15 per cent was added. In each case, the hemolysate representing the cells hemolyzed by the salt strength used, but not by the previous salt strengths, was collected and assayed for hemoglobin, methemoglobin and radioactivity. The relatively few unhemolyzed cells remaining in the supernatant (because of the slow centrifuge speed used to avoid damage to cells) were hemolyzed by the addition of saponin. Serial osmotic lysis was carried out on the blood of T.P. using a higher centrifuge speed (680 G) to remove all suspended erythrocytes. Radioactivity determinations were carried out on 7.00-ml aliquots in a Nuclear-Chicago model DS 3 scintillator detector, model 1810 spectrometer, and model 161 A scaling unit.

G. Erythrocyte survival studies. Cr^{51} survival studies were carried out by adding sodium chromate (Rachromate, Abbott Laboratories) in 100 to 200 μc quantities to approximately 30 ml of blood. The plasma was removed after centrifugation and the cells were washed once in isotonic saline. Radioactivity determinations were carried out with the same equipment used for counting the Fe^{59}. The blood volume was assumed to remain constant during the red cell survival studies and, therefore, no corrections for changes in hematocrit were made.

RESULTS

A. In vitro studies

1. Sickling-oxygen tension curves. Figures 1 and 2 demonstrate the effect of various concentra-

[3] We appreciate the generous gift of this material.

tions of methemoglobin induced *in vivo* by the administration of sodium nitrite (Figure 1) or PAPP (Figure 2) on the distorting effects of low oxygen tensions on sickle cells. It is noted that there is well marked inhibition of red cell distortion. The criteria used for assessing distortion of red cells do not materially effect the relative position of the curve.

2. Oxygen dissociation curve of red cells containing methemoglobin. The percentage saturation with oxygen of red cells of Subject T.P. before and after methemoglobinemia was induced by PAPP is depicted in Figure 3. The presence of less than 25 per cent methemoglobin in red cells

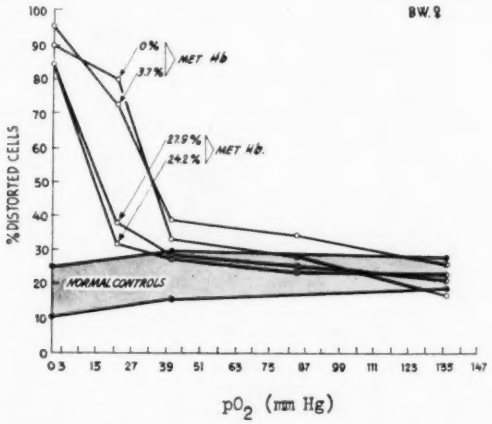

FIG. 1. EFFECT OF METHEMOGLOBINEMIA INDUCED *IN VIVO* BY SODIUM NITRITE ADMINISTRATION ON THE SICKLING-OXYGEN TENSION CURVE OF ERYTHROCYTES. At low oxygen tensions there is less distortion of cells containing 24.2 and 27.9% methemoglobin than in those containing zero or 3.7% methemoglobin.

apparently exerts little effect on the dissociation of oxygen from oxyhemoglobin. Darling and Roughton (19), using normal red blood cells, have previously demonstrated that at low levels of methemoglobin the shift in the oxygen dissociation curve is slight. At the relatively low levels of methemoglobin used in our studies the shift in the oxygen dissociation curve might well have been too small to be detected in the limited number of measurements which we have made, if it occurred at all. Furthermore, in our calculations, the amount of hemoglobin available for dissociation has been estimated by subtracting the amount of methemoglobin present from the total hemoglobin

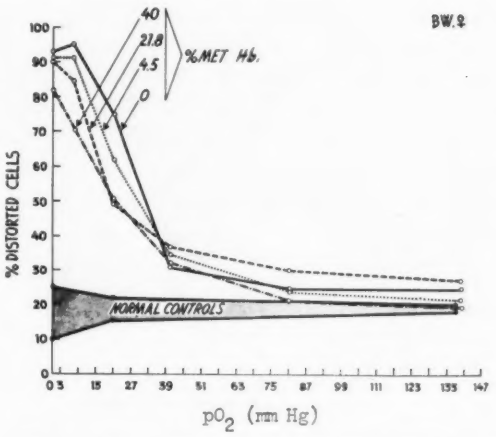

FIG. 2. EFFECT OF METHEMOGLOBINEMIA INDUCED *IN VIVO* BY PAPP ON THE SICKLING-OXYGEN TENSION CURVE OF ERYTHROCYTES. At low oxygen tensions there is successively less distortion of cells as the amount of methemoglobin in the cells increases.

as determined by the cyanmethemoglobin method. If any additional pigments (hemoglobin degradation products) contributing to optical density but not capable of carrying oxygen, such as sulfhemoglobin, were present, these would give a falsely high estimation of the amount of hemoglobin pres-

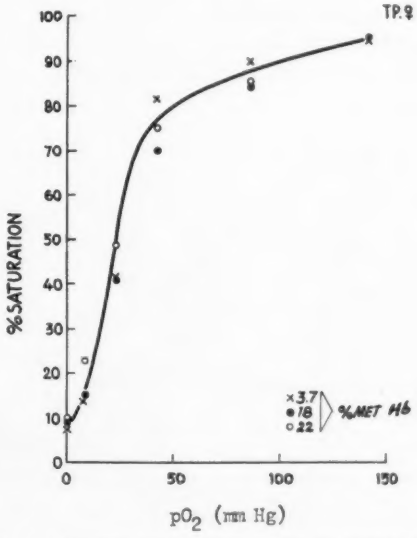

FIG. 3. OXYGEN DISSOCIATION CURVE OF SICKLE CELLS AT HIGH AND NEAR NORMAL METHEMOGLOBIN LEVELS. Methemoglobinemia was induced *in vivo* by the administration of PAPP. No significant change in the dissociation curve could be demonstrated.

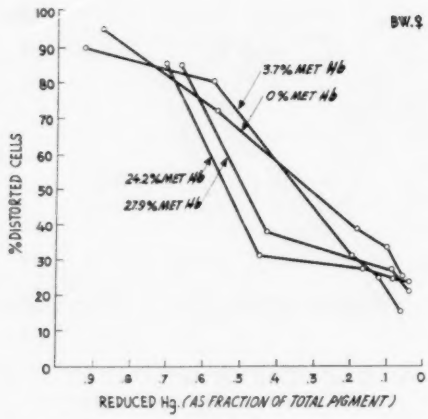

FIG. 4. EFFECT OF METHEMOGLOBINEMIA INDUCED *IN VIVO* BY SODIUM NITRITE ADMINISTRATION ON THE SICKLING-REDUCED HEMOGLOBIN CURVE OF ERYTHROCYTES, AS DERIVED FROM FIGURES 1 AND 3. The fraction of the total pigment in the form of reduced hemoglobin has been computed and appears as the abscissa. When expressed in this way, the percentage of cells distorted seems relatively independent of the percentage of methemoglobin in the red cells.

ent. In this way, the calculated oxygen dissociation curve would tend to be displaced to the right. This might obscure any slight leftward shift caused by the presence of methemoglobin. In agreement with the data of others (20–22), the oxygen dissociation curve of sickle cells appears to be somewhat to the right of that usually presented for normal red blood cells.

3. Reduced hemoglobin-red cell distortion curves. Although, as shown in Figure 3, the presence of methemoglobin exerts relatively little effect on the dissociation of oxygen from oxyhemoglobin, the amount of the hemoglobin in each red cell participating in the hemoglobin-oxyhemoglobin equilibration is diminished when some of it has been converted to methemoglobin. To determine whether the presence of methemoglobin itself exerts an effect on the sickling process, or whether it exerts its action chiefly by removing hemoglobin from the $Hb \rightleftarrows HbO_2$ equilibrium, the curves correlating red cell distortion and oxygen tension have been

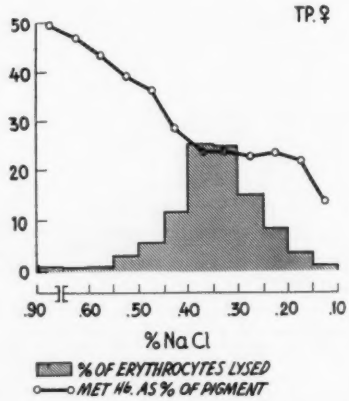

FIG. 6. RELATIONSHIP BETWEEN CELL AGE AND METHEMOGLOBIN CONTENT OF THE ERYTHROCYTES AS STUDIED BY FRACTIONAL OSMOTIC LYSIS. The cells most susceptible to lysis (presumably the older red cells) contain the greatest amount of methemoglobin.

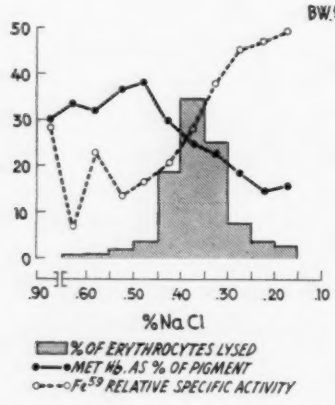

FIG. 5. RELATIONSHIP BETWEEN CELL AGE AND METHEMOGLOBIN CONTENT OF THE ERYTHROCYTES AS STUDIED BY FRACTIONAL OSMOTIC LYSIS. The cells most susceptible to lysis (presumably the older red cells) contain the greatest amount of methemoglobin.

replotted to compare the proportion of the cells distorted with the concentration of reduced sickle hemoglobin in each cell. The reduced sickle hemoglobin concentration in the cell has been estimated by multiplying the percentage of reduced hemoglobin, as read from the oxygen dissociation curve (Figure 3), by the fraction of hemoglobin not converted to methemoglobin and, therefore, available for dissociation. The curves obtained show that the percentage of cells distorted is closely related to the reduced sickle hemoglobin concentration and is not greatly influenced by the presence of methemoglobin. Representative curves obtained by replotting data from Figure 1 are presented in Figure 4.

B. The effect of cell age on methemoglobin levels

Ten μc of Fe^{59}-citrate was given intravenously to Subject B.W. Maximal blood levels were achieved after 7 days. Nine days after the administration of Fe^{59}, the patient was given 200 mg of PAPP. One and one-half hours later, the blood methemoglobin level was approximately 40 per cent. Blood was drawn and the red cells were fractionated by differential osmotic hemolysis. The supernatant solutions were analyzed for amount of hemoglobin and the percentage of hemoglobin which was methemoglobin. These data are presented in Figure 5. In agreement with the data of Marks and Johnson (23) and of Levy,

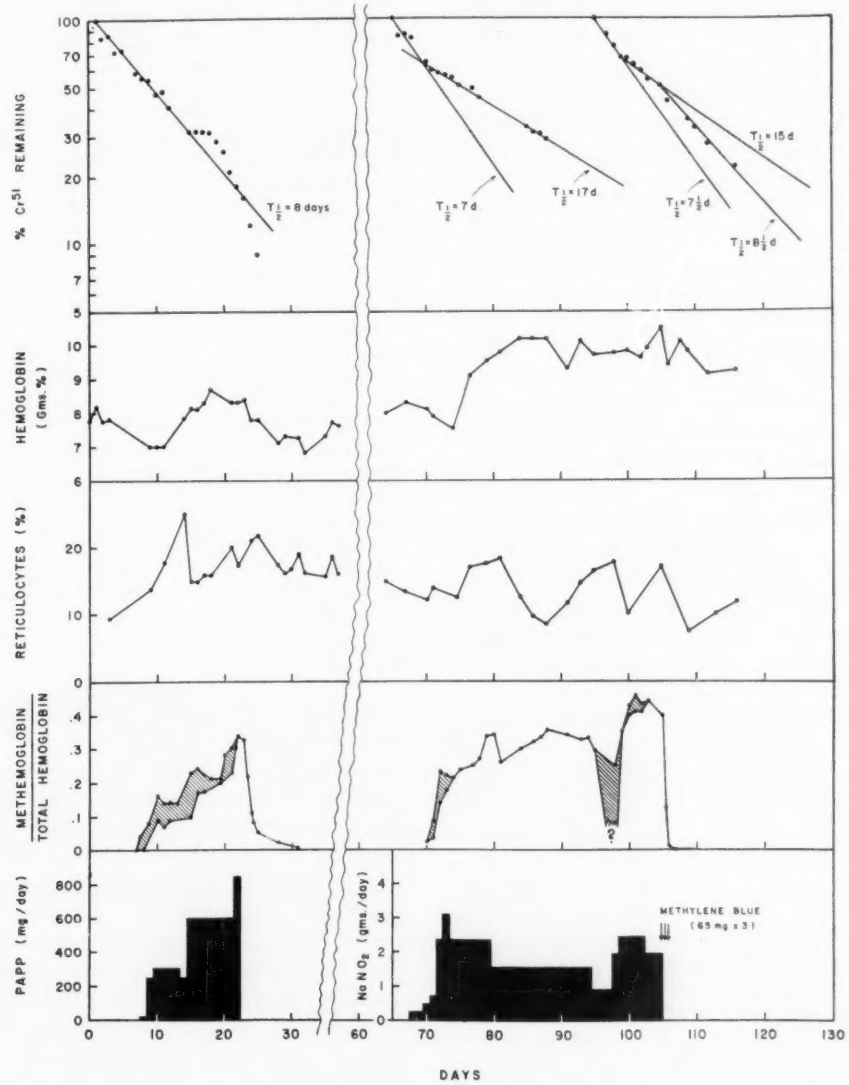

FIG. 7. EFFECT OF PAPP AND SODIUM NITRITE ADMINISTRATION TO L.A. ON THE METHEMOGLOBIN CONTENT OF THE BLOOD, RETICULOCYTE COUNT, HEMOGLOBIN CONCENTRATION OF THE BLOOD AND Cr^{51} RED CELL SURVIVAL. The shaded areas in the methemoglobin panel of Figures 7–11 represent the range during the day when estimated from multiple daily determinations.

Walter and Sass (24) on normal erythrocytes, the younger members of the sickle cell population, those labeled with Fe^{59}, are most resistant to osmotic lysis. The slightly elevated Fe^{59} levels of the 0.65 and 0.55 per cent fractions may be due to the unusual fragility of a portion of the young cells, as was found in normal blood by Levy and co-workers (24), or to incomplete sedimentation of some reticulocytes at the relatively slow centrifuge speeds used. The younger red cell fractions contain the least methemoglobin. Very little radioiron was found in the 0.60, 0.50 and 0.45 per cent fractions and the maximal methemoglobin levels were encountered in these fractions. To determine whether the larger amounts of methemoglobin in the most easily lysed fractions were really a function of increased susceptibility of methemoglobin-containing cells rather than of cell age, differential osmotic hemolysis was repeated on the following day, after the methemoglobin concentration had returned to zero. There was no change in the susceptibility of the erythrocytes to osmotic lysis, indicating that the presence of methemoglobin does not increase the susceptibility of red cells to osmotic lysis. Two weeks later, the patient was again given 200 mg PAPP. This time the Fe^{59}-containing cells had aged somewhat and more radioactivity was found in the least resistant fractions. The pattern of methemoglobin formation was the same. Differential osmotic hemolysis studies were also carried out on the red cells of another patient, T.P., who had received PAPP for several weeks. A higher centrifuge speed was used to clear all remaining cells from the supernatant (see Methods). The relationship of the percentage of hemoglobin in the form of methemoglobin in various fractions as determined by this experiment is presented in Figure 6.

C. Clinical observations

1. Subject L.A. (Figure 7). The red cells of Subject L.A. were labeled with Cr^{51} and their survival followed for a baseline period of 7 days. PAPP was then administered at 4-hour intervals in gradually increasing doses, as shown in Figure 7. The percentage of methemoglobin increased concurrently. When the methemoglobin level reached approximately 20 per cent of the total pigment there was a striking increase in the survival of the red blood cells as estimated by the Cr^{51} method. This lasted for only a few days. In spite of continued drug administration and a rising level of methemoglobin to a maximum of approximately 33 per cent of the total pigment, red cell destruction soon became accelerated and exceeded the baseline rate. This suggested that PAPP, an aromatic amine, was in itself hemolytic. Concurrently with the methemoglobinemia and lengthening of red cell survival, there was a modest reticulocytosis and a rise in the total hemoglobin level of the blood.

The patient's erythrocytes were rechromated approximately 1 month after the conclusion of the first study and methemoglobinemia was induced again, this time by administering gradually increasing doses of sodium nitrite. No toxic side effects from sodium nitrite were encountered, but the methemoglobin level rose to between 20 and 30 per cent of the total pigment. The baseline Cr^{51} half-time was 7 days, closely comparable with the 8-day $T_{\frac{1}{2}}$ obtained on the patient in the previous study. During the period of methemoglobinemia, the Cr^{51} survival was lengthened to a $T_{\frac{1}{2}}$ of 17 days. There was no distinct change in the reticulocyte count and the total hemoglobin level of the blood rose. While still receiving sodium nitrite, the patient's erythrocytes were rechromated. Unfortunately, at this time the nighttime doses of nitrite were omitted for several days. It is not known to what level the methemoglobin concentration of the blood fell during this time. The Cr^{51} $T_{\frac{1}{2}}$ was 7.5 days. After reinstitution of sodium nitrite therapy in full doses, the $T_{\frac{1}{2}}$ was lengthened to 15 days. Then nitrite administration was discontinued and three 65-mg doses of methylene blue were administered orally, quickly lowering the methemoglobin level. Red cell destruction became somewhat accelerated with a $T_{\frac{1}{2}}$ of 8.5 days, closely comparable to the baseline levels of 8 and 7 days which were observed in the two previous Cr^{51} studies.

The patient had no acute symptoms at any time during the study and no change was noted in her general clinical status, serial electrocardiograms, urinalysis, or in her capacity to concentrate urine after water deprivation.

Comment. The administration of PAPP appeared to result in prolongation of red blood cell survival as estimated by the Cr^{51} method, but subsequently there was accelerated red cell destruction. The administration of sodium nitrite was associated with a more sustained prolongation of red cell survival. The possibility has been considered that the apparent prolongation of red cell survival in the second study was an artifact, produced by a heterogeneous red cell population comprised of short-lived and long-lived cells. This seems unlikely, since in the early portion of the first study there was no indication that there was a two-component population. However, a further attempt was made to rule out this possibility and to determine whether prolongation of red cell-life span induced by sodium nitrite occurred in all

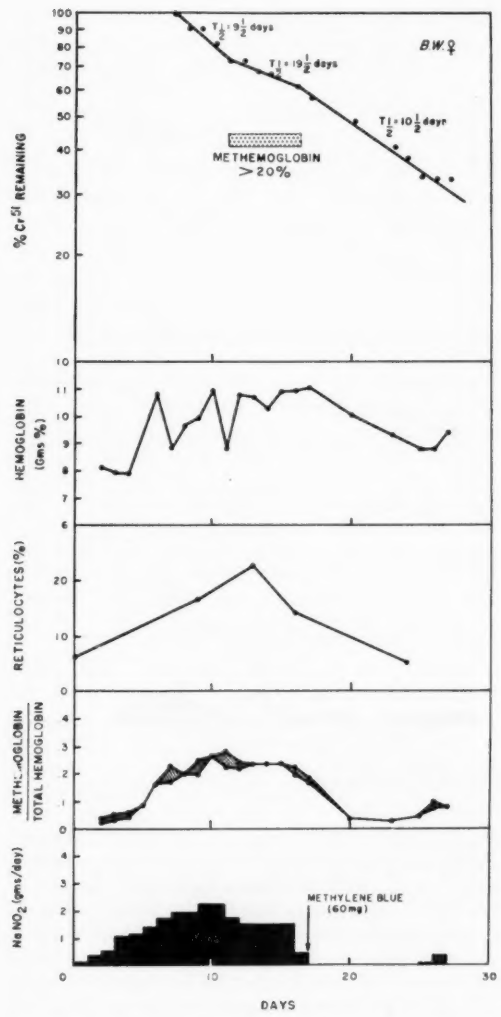

FIG. 8. EFFECT OF SODIUM NITRITE ADMINISTRATION TO B.W.

members of the red cell population or only in the older cells (which contain a greater proportion of methemoglobin). Interpretation of this experiment is hampered by failure of the patient to receive nighttime doses of sodium nitrite. However, the third Cr^{51} study clearly demonstrates that the changes in the first two studies were not due merely to a mixed red cell population consisting of a long-lived and a short-lived component. It also shows that prolongation of red cell life span can be achieved, even in relatively young cells, when methemoglobinemia is induced by sodium nitrite administration.

It is impossible to determine whether the rise in the hemoglobin level which was observed was chiefly due to "driving" of the marrow as a result of the decreased oxyhemoglobin levels or to the decreased destruction of erythrocytes. Probably both mechanisms play a role, but the latter is emphasized in a later study carried out on T.P. (see below).

2. Subject B.W. (*Figure 8*). B.W. was given gradually increased doses of sodium nitrite. Her red cells were chromated when her methemoglobin level was already between 15 and 20 per cent of the total pigment (Figure 8). Before the level of methemoglobinemia was consistently greater than 20 per cent, the $T_{\frac{1}{2}}$ was 9.5 days, as computed by the method of least squares (25). A $T_{\frac{1}{2}}$ of 19.5 days was found when a curve was fitted to the points obtained after methemoglobin levels above 20 per cent of the total pigment were consistently achieved. Upon withdrawal of sodium nitrite and administration of methylene blue to hasten the conversion of methemoglobin to hemoglobin, red cell survival was for the next 8 days shortened to a $T_{\frac{1}{2}}$ of 10.5 days, closely comparable to the original $T_{\frac{1}{2}}$ of 9.5 days.

No unusual clinical symptoms were observed. However, approximately 1 week after the methemoglobin level had returned to zero, the patient developed a mild, painful crisis. This seemed to provide an opportunity to study the effect of methemoglobin production on this complication. Small doses of sodium nitrite were administered. There was an apparent lengthening of red cell survival, although since the observations made were scanty, their significance remains somewhat doubtful. The painful symptoms subsided in approximately 24 hours. Relief of pain was not impressive. Several months later, we were presented with another opportunity to observe the patient during a painful crisis. The results of administration of a single dose of PAPP were approximately the same. Rapid induction of methemoglobinemia of 29 to 36 per cent for 1 hour did not seem to affect the pain, which subsided after about 48 hours. No attempt to induce prolonged methemoglobinemia was made.

Comment. This study confirms that prolongation of survival of even relatively young erythrocytes can be achieved when sodium nitrite is administered. Efforts to influence painful crises yielded disappointing results, although such results are difficult to evaluate because of the marked variability in the natural history of painful crises.

3. Subject T.P. (*Figure 9*). The red cells of T.P. were labeled with Cr^{51} and baseline survival studied for several days. Methemoglobinemia was induced with PAPP on two occasions—first for 6, then for 7 days.

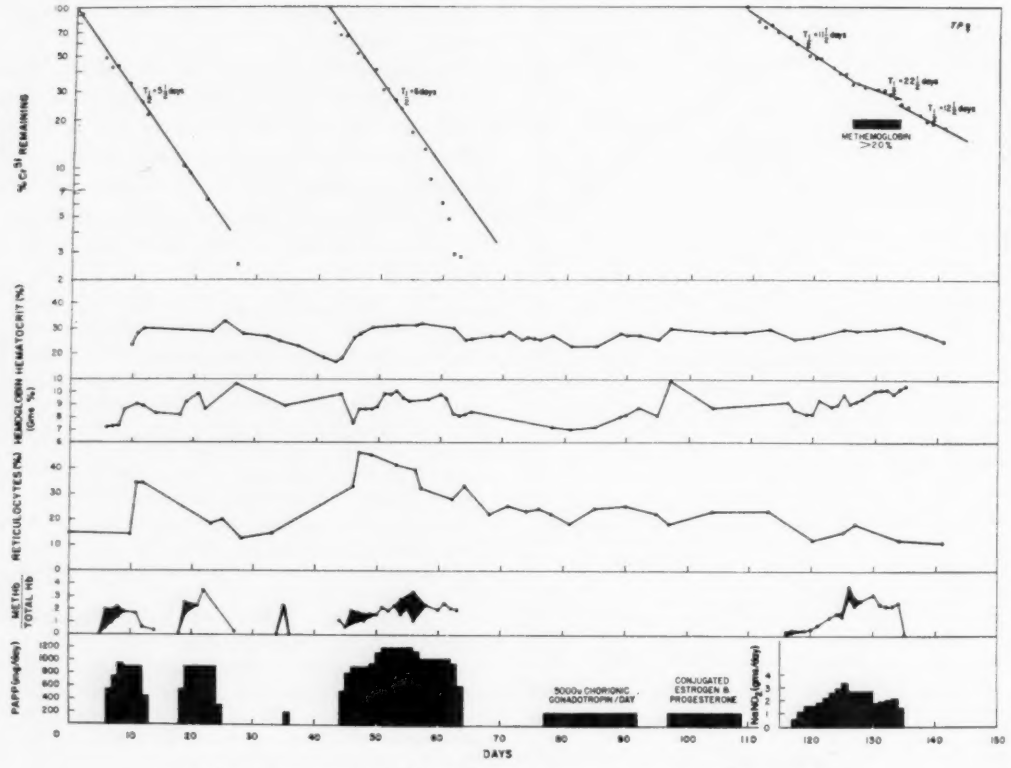

FIG. 9. EFFECT OF PAPP AND SODIUM NITRITE ADMINISTRATION TO T.P.

No significant alteration of Cr^{51} survival was observed. The reticulocyte count and hematocrit rose slightly, and hemoglobin showed a questionable increase. Two weeks following the second course of PAPP administration, the patient developed chest pain, fever, and a pulmonary infiltrate of the right base. She was treated with anticoagulants for the next 2.5 weeks, and her symptoms and signs quickly disappeared. The patient's red cells were again labeled with Cr^{51} and a more prolonged course of treatment with PAPP lasting 19 days was carried out. There was only questionable, transient prolongation of Cr^{51} survival lasting for a few days and then accelerated destruction, as had been observed in the study carried out on L.A. However, there was a well marked rise in the hematocrit, not accompanied by a rise in the hemoglobin. Thus, the patient's mean corpuscular hemoglobin concentration fell. This apparently anomalous finding was easily explained when her red cells were examined for Heinz bodies. Most of the cells contained these particles, probably consisting of denatured protein (*26*). The patient was next given a short course of injections with chorionic gonadotropin in an effort to influence fetal hemoglobin synthesis. The results were equivocal: a rapid rise of alkali-resistant hemoglobin was followed by a return to near baseline level while hormone was still being administered. The administration of conjugated equine estrogens and progesterone resulted in no change in alkali-resistant hemoglobin levels.

A third Cr^{51} survival study was carried out to determine whether methemoglobinemia induced by nitrite would prolong red cell survival in this patient as it had in Subjects L.A. and B.W. The baseline Cr^{51} survival time was longer than that found in the two earlier studies, with a $T_{\frac{1}{2}}$ of 11.5 days, as computed by the method of least squares (*25*). This prolongation may be explained by the fact that the patient had just experienced accelerated red cell destruction as a result of PAPP administration. Drug-induced hemolytic anemia affects chiefly the older members of the red cell population (*27*); thus, prolonged survival may be expected following drug-induced hemolysis. When sodium nitrite was administered to the patient the Cr^{51} survival time remained unchanged, with a computed $T_{\frac{1}{2}}$ of 11.5 days, until the methemoglobin level consistently exceeded 20 per cent of the total pigment. The slope of the survival curve then changed to give a computed $T_{\frac{1}{2}}$ of 22.5 days. After sodium nitrite administration was discontinued the slope of the survival curve returned to 13 days, comparable with that of the period before sodium nitrite administration. During the course of the study the patient's leg

ulcers healed rapidly. There was no change in her capacity to concentrate urine after water deprivation and no change in her general clinical condition.

Comment. In this patient, the hemolytic effect of protracted PAPP administration was confirmed. It was again demonstrated that sodium nitrite could prolong red cell survival at methemoglobin levels at which no significant prolongation was achieved with PAPP. The healing of the leg ulcers could probably be attributed as easily to the extended period of hospitalization as to any beneficial effects from the administration of methemoglobin-forming agents.

4. Subject J.G. (Figure 10). The red cells of J.G. were labeled with Cr^{51} and she was given gradually increased doses of sodium nitrite every 4 hours. After 9 days, the patient was receiving 390 mg sodium nitrite every 4 hours and complained of headache. However, methemoglobin levels were only about 11 per cent. Therefore, the dosage of sodium nitrite was increased by one-third to 520 mg for one dose. When this dose failed to produce any further symptoms and the methemoglobin level was still well below 20 per cent, an additional dose of 260 mg sodium nitrite was given after 2 hours. Two hours later there were no symptoms, although the blood pressure was 100/0. Another 520-mg dose of sodium nitrite was administered. About 1.5 hours later, the patient complained of substernal pain which lasted for about 10 minutes and disappeared when oxygen was given. The methemoglobin level at the time of pain was only 22 per cent of the total pigment. An electrocardiogram taken shortly after the pain subsided showed no change from the previous tracing, except for a more rapid rate and the appearance of supraventricular extrasystoles. An EKG taken 3 days later was entirely normal. The dosage of sodium nitrite was decreased to 390 mg every 4 hours. There were no further symptoms and the dose was increased to 455 mg, at which time the patient again developed an episode of chest pain, which subsided in a few minutes after administration of oxygen and codeine. The methemoglobin level at this time was only 19 per cent. Further administration of sodium nitrite to J.G. was abandoned. In the course of the study there were no changes in serial urinalysis, serum bilirubin determinations, or capacity to concentrate urine after water deprivation.

Taken as a whole the Cr^{51} survival curve does not deviate significantly from the baseline curve. The minor fluctuations which occurred do not coincide either with changes in nitrite dosage or with the methemoglobin level and are not readily explained.

Comment. This subject had the most severe degree of anemia of any of the patients studied, and consequently her red cells have the youngest average age. Probably because the methemoglobin-reducing systems of young cells are more active than those of older red cells (28), it was impossible to achieve adequate levels of methemoglobinemia in this patient without encountering alarming toxic side effects from sodium nitrite.

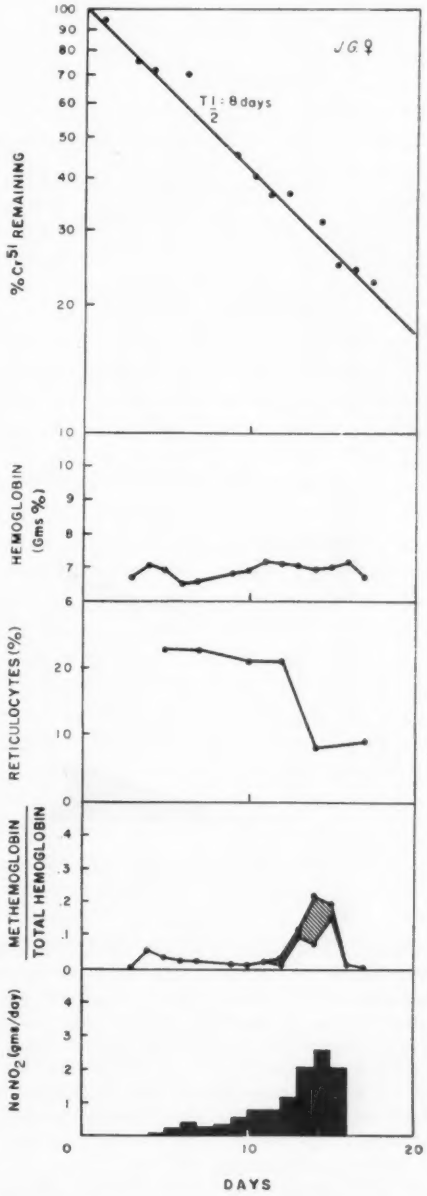

FIG. 10. EFFECT OF SODIUM NITRITE ADMINISTRATION TO J.G.

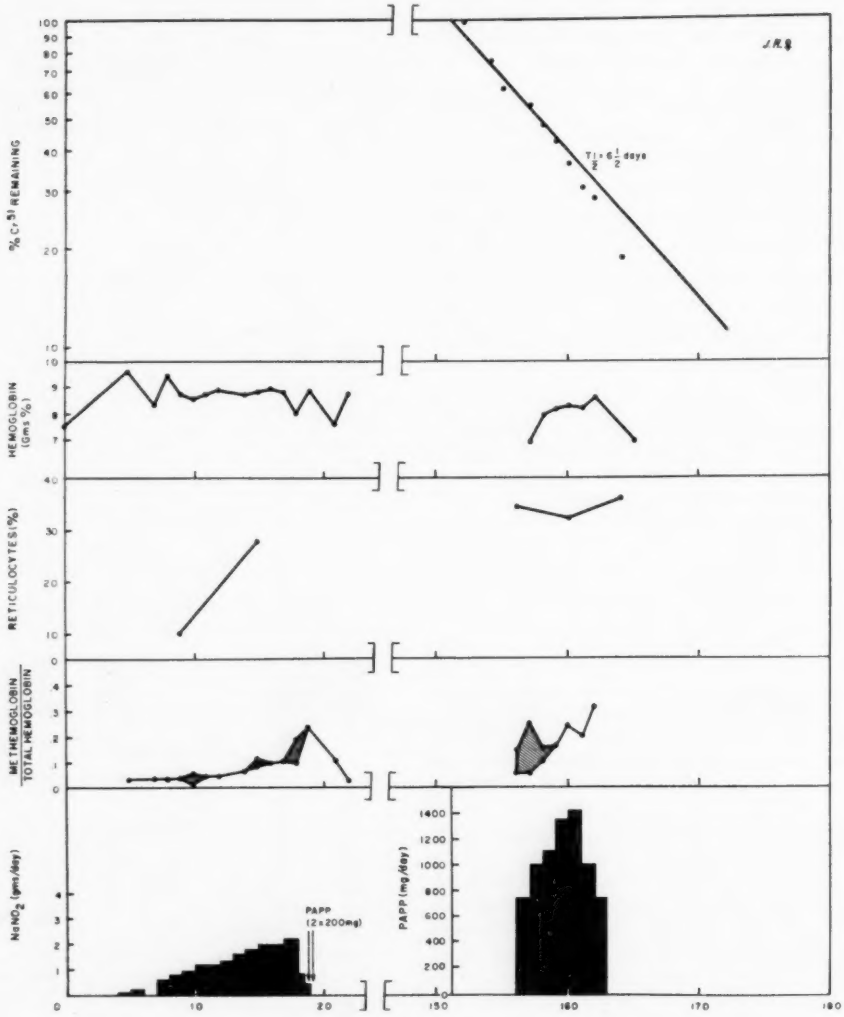

FIG. 11. EFFECT OF PAPP AND SODIUM NITRITE ADMINISTRATION TO J.R.

5. Subject J.R. (Figure 11). J.R. was given gradually increased doses of sodium nitrite which resulted in a gradual rise in her methemoglobin level. When the dosage had reached 325 mg every 4 hours, the patient experienced epigastric burning but the methemoglobin level remained at approximately 10 per cent of the total pigment. It was therefore deemed wise to discontinue sodium nitrite administration. A Cr^{51} survival curve was, unfortunately, unsatisfactory because the red cells had been washed with a sodium chloride solution containing 0.9 per cent benzoic acid as a preservative. Subsequent investigations demonstrated that benzoic acid at this concentration damages red cells, increasing their osmotic fragility and causing, upon injection, their almost immediate removal from the circulation. A second study was initiated in which PAPP was administered. Methemoglobinemia of between 20 and 30 per cent was achieved without any untoward symptoms. There was no prolongation of Cr^{51} survival, and indeed, there was accelerated destruction of red cells within a few days' time after PAPP administration had been initiated. There was no change in the patient's general clinical status, serial electrocardiograms, urinalysis, bilirubin level or capacity to concentrate urine on water deprivation. Two days after discharge from the hospital, the patient developed an episode of nausea, vomiting and

diarrhea which subsided spontaneously within about 24 hours, and which was considered to be unrelated to either her sickle cell disease or the experimental therapy. Several months after these studies were conducted, this patient was also given a course of chorionic gonadotropin, 10,000 U daily. A slight rise in alkali-resistant hemoglobin was observed, but a higher level had been observed previously while the patient was receiving no hormone therapy.

Comment. Some degree of accelerated red cell destruction in a patient receiving PAPP was again demonstrated.

DISCUSSION

It is usually assumed that the clinical manifestations of sickle cell disease are due to the abnormal physicochemical properties of sickle hemoglobin, which result in the distortion of the red cells when the oxygen tension is reduced. It must be admitted, however, that although it is reasonable to assume that sickling of red cells is responsible for the clinical course of sickle cell disease (29), this assumption can by no means be considered proven. There are discrepancies between the number of sickled cells and the severity of the clinical disease, and it has been suggested that other disturbances of red cell function may be important clinically (4). Nonetheless, it might be possible to influence favorably the course of this disease by changing the composition of hemoglobin within the red cells in such a way that the extent of sickling would be diminished. An ideal solution to the problem of sickling of erythrocytes would be to induce synthesis of type A hemoglobin to take the place of synthesis of type S hemoglobin. This is technically beyond our reach today, for the patient with sickle cell disease does not have the genetic potential for the synthesis of type A hemoglobin and changing the genetic constitution of human tissues is not yet feasible.

A second, probably almost as satisfactory solution, might be to induce synthesis of large amounts of type F hemoglobin in the red cells of patients with sickle cell disease in the place of hemoglobin S. Patients with sickle cell disease do have the potential for synthesis of the gamma chain of the hemoglobin molecule, and indeed normally produce increased amounts of fetal hemoglobin (30). Fetal hemoglobin interferes with the sickling process in infants (10) and in hereditary persistence of fetal hemoglobin (31). As yet, no method for increasing the synthesis of F hemoglobin is known, although sensitivity in the ratio between hemoglobin F and hemoglobin A synthesized to oxygen tension has been demonstrated (32, 33). The alteration of this ratio is due, however, to the fact that while both hemoglobin F and hemoglobin A synthesis is inhibited by anoxia, the degree of inhibition is greater in the case of hemoglobin A. The observation (34) that cyanotic congenital heart disease does not appear to be associated with the appearance of hemoglobin F suggests that anoxia is not an important regulatory factor. It has also been observed, by using alkali denaturation and immunologic technique (35, 36), that there is increase in fetal hemoglobin content of the mother's blood during the second trimester of normal pregnancy and during molar pregnancy. This has not been confirmed with sensitive measurements using the tryptophan fine-line spectrum after alkali denaturation (34). Nonetheless, the possibility that the hormonal environment might influence fetal hemoglobin synthesis appeared to us to warrant further investigation. A transient rise in alkali-resistant hemoglobin was observed in two patients following chorionic gonadotropin administration, but its significance is as yet uncertain. Further studies are obviously necessary. This problem is currently under investigation in our laboratory.

A third approach is to modify the hemoglobin within the red cell chemically without attempting to influence the type of hemoglobin which is synthesized by the red cell precursor. In this paper, the potential value of converting sickle hemoglobin to methemoglobin is examined. An ideal chemical modification of hemoglobin would be such that: *1*) the modified compound would continue to carry oxygen and carbon dioxide normally, *2*) its modification would be induced by the administration of nontoxic agents, *3*) infrequent drug administration by the oral route would suffice to produce the desired change in hemoglobin in a reliable fashion with a minimum of laboratory control, *4*) sickling would be effectively inhibited even at relatively low oxygen tensions. It is obvious that drug-induced methemoglobinemia falls seriously short in several respects. Frequent administration of drugs with certain toxic side effects with appropriate laboratory controls is required to maintain an adequate level of methemoglobin in the blood.

Methemoglobin does not carry oxygen. However, it does, as we have confirmed in this study, effectively inhibit the sickling process. Substantial inhibitions of sickling can be achieved with a compromise of only about 20 per cent of the oxygen-carrying capacity of the blood. In agreement with the conclusions drawn from studies in experimental animals by Jalavisto and associates (37–39), and in human red cells by Jung (40) and Waller, Schlegel, Müller and Löhr (41), the methemoglobin concentration of the older red cells was greater than that of the younger members of the red cell population. Thus, very marked inhibition of sickling of some of the cells may have been achieved. The current studies shed some light on the mechanisms of inhibition of sickling by methemoglobin. It would appear that methemoglobin exerts its chief effect by removing pigment from the equilibrium between reduced hemoglobin and oxyhemoglobin rather than by interfering with the sickling process in a manner analagous to the interference produced by fetal hemoglobin (10). In effect, it renders the red cell hypochromic with respect to hemoglobin, thus reducing the absolute amount of reduced sickle hemoglobin present at a given oxygen tension and diminishing the tendency of the red cell to be deformed.

Regardless of the mechanism of the effect of methemoglobin in inhibiting sickling, the cautious trial of methemoglobin-forming agents in the treatment of sickle cell disease seemed to us to be justified. Many treatments of sickle cell disease have been advocated in the past; none is considered satisfactory (5). To our knowledge, the studies we have reported here represent the first experimental therapy which has been shown to prolong red cell survival. The effect of inducing methemoglobinemia with PAPP and sodium nitrite has been investigated in five patients with sickle cell disease. When methemoglobin levels above 20 per cent were produced with sodium nitrite there was a consistent prolongation of red blood cell survival. At lower methemoglobin levels, no such prolongation of red cell survival was observed. PAPP failed to produce any sustained significant prolongation of red cell survival, even at methemoglobin levels considerably higher than those achieved with sodium nitrite. This is in all likelihood due to the fact that PAPP, an aromatic amine, is itself hemolytic. The possibility has been considered that sodium nitrite produces prolongation of red cell survival by a mechanism independent of the methemoglobinemia which it induces. Sodium nitrite is known to have marked effects on the vascular system, and it is conceivable that these may be responsible, in part, for its effect in prolonging red blood cell survival. However, the patient who failed to develop significant degrees of methemoglobinemia, even with large amounts of sodium nitrite (J.G.), failed to experience sustained prolongation of red blood cell survival.

Methemoglobinemia was well tolerated by all of the patients. This was true even when relatively large amounts of methemoglobin were formed in a brief period of time. This is consistent with the point of view that the clinical manifestations of sickle cell disease are not due primarily to the anemia. Neither could any clinical benefit be ascribed to the induction of methemoglobinemia, nor was the renal concentrating defect influenced. The latter finding is not surprising in view of the fact that the renal defect of patients with sickle cell disease is only reversible in early childhood (42).

It has been known for some time that tolerance to the side effects of nitrites develops upon continued exposure. Thus, it has been pointed out that munitions factory workers may suffer from nitrite-induced headaches during the first few days of employment. They are subsequently asymptomatic unless they leave work for several days. Upon return to work they often develop headaches again. Workers have learned to avoid these symptoms by continuing to expose themselves to nitrite by placing a small quantity of nitroglycerin in their hatbands so that it will be absorbed through the skin (43). There is conflicting evidence as to whether tolerance can be induced to sodium nitrite (44, 45), although there is general agreement about other nitrites. Nonetheless, we were able to administer large doses of nitrite to most of our patients by using gradually increased doses without encountering side effects. This was not true in all cases, however. One patient (J.G.) developed alarming symptoms when the sodium nitrite dosage was increased rapidly, and symptoms recurred at the same level of nitrite even when the dosage was increased slowly. Another

patient (J.R.) developed symptoms which were sufficiently annoying to make it seem advisable to discontinue therapy. Aside from these toxic side effects of nitrite administration, it is obviously impractical to administer any drug to patients every 4 hours, day and night, over a long period of time. If methemoglobinemia were to have any practical value in the long-term therapy of sickle cell disease, it would have to be induced by a preparation which could be administered at much less frequent intervals. On the other hand, it is conceivable that short-term methemoglobinemia might be of value in the treatment of crises. We have hesitated to give sodium nitrite in the treatment of crises, however, since the tolerance to the vascular effects is variable. Administration of enough nitrite to result in formation of methemoglobin levels sufficient to affect the sickling phenomenon might result in serious side effects. PAPP, on the other hand, might have some promise in the therapy of crises, since high levels of methemoglobin can be induced within less than an hour by the administration of this compound. Our limited experience with the use of this material in the treatment of painful crises has been disappointing. Since the course of crises is variable, a more extensive controlled trial would be necessary to determine whether the administration of this compound is of value in crises.

SUMMARY

1. The induction of methemoglobinemia *in vivo* by the administration of para-aminopropriophenone (PAPP) or of sodium nitrite produced a shift to the left in the sickling-oxygen tension curve of the red cells of patients with sickle cell disease.

2. Conversion of 20 per cent of the sickle cell hemoglobin to methemoglobin produced such an effect, but did not materially influence the oxygen dissociation curve of the intact red blood cells.

3. Most or all of the effect of methemoglobin in inhibiting sickling could be explained on the basis of removal of pigment from the oxyhemoglobin-reduced hemoglobin equilibrium, resulting in a lower concentration of reduced sickle hemoglobin at a given oxygen tension.

4. More methemoglobin was present in the older members of the red cell population than in young red cells of patients with sickle cell disease after administration of PAPP.

5. When sodium nitrite was administered to patients with sickle cell disease and the methemoglobin concentration of the blood reached levels greater than 20 per cent of the total pigment, there was an increase in the survival of red blood cells, as estimated by the Cr^{51} method. There was also a tendency for the hemoglobin content of the blood to rise.

6. When methemoglobinemia was induced in patients with sickle cell disease by the administration of PAPP, there was in at least one case transient prolongation of red blood cell survival as estimated by the Cr^{51} method. In all cases in which PAPP was administered for more than a few days, there was eventually accelerated destruction of red cells as estimated by this method. Administration of PAPP was sometimes associated with an increase of the hematocrit not accompanied by increase in hemoglobin concentration of the blood. This was attributed to denaturation of some of the hemoglobin within the red cells to Heinz bodies.

7. In none of the patients was there any substantial change in clinical status during drug administration, although alarming side effects were noted in one patient at high dosages of sodium nitrite and another patient noted troublesome side effects from sodium nitrite administration. Preliminary attempts to treat painful crises of patients with sickle cell disease by the administration of sodium nitrite yielded unimpressive results.

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ANTIBODY PRODUCTION IN RHEUMATIC DISEASES. THE EFFECT OF BRUCELLA ANTIGEN*

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The possibility that a disordered immune response is related to the development of the rheumatic diseases has been an attractive hypothesis for investigation for years. Several investigators have attempted to demonstrate quantitative differences in the immune response between patients with these diseases and control subjects when they were inoculated with various antigens. Rantz, Creger and Choy (1) challenged patients with rheumatic fever and rheumatoid arthritis by injecting isologous red blood cells intravenously, and by the inoculation of influenza A and B vaccine. The patients with rheumatic fever responded with an increased titer of isohemagglutinins and in increased titer of antibodies to influenza virus compared with the control groups. There was no difference in the immune response to these two antigens between the rheumatoid arthritic patients and control subjects. In a prospective study, Rejholec (2) administered brucella vaccine subcutaneously to a group of 900 children. A small group developed very high titers of brucella antibody, measured by the indirect Coombs technique. All the children were followed clinically and a very high percentage of the hyper-reactors were shown subsequently to develop rheumatic fever; indeed, the only cases of rheumatic fever appeared in this hyper-reactor group. Miller, Kibrick, and Massel (3), by using influenza virus and typhoid O and H antigens, were unable to show hyper-reactivity in a group of patients with rheumatic fever. Other studies with pneumococcal polysaccharide by Quinn, Seastone and Dickie (4) and with diphtheria toxoid by Kuhns and McCarty (5) similarly showed no hyper-reactivity.

The present studies were initiated in the face of these conflicting data and with the following two objectives in mind: *1*) to determine whether differences in antibody response to an antigenic stimulus of a nonstreptococcal, presumably unfamiliar antigen could be demonstrated between control patients and patients with various rheumatic diseases; and *2*) to determine whether challenge by a specific heterologous antigen might influence one or another of the abnormal antibodies or antibody-like substances circulating in the blood of patients with rheumatic diseases.

MATERIALS AND METHODS

Nineteen patients with rheumatoid arthritis in all stages of activity, 11 patients with systemic lupus erythematosus, 5 patients with acute rheumatic fever, and 6 patients with acute glomerulonephritis formed the study group. Almost all patients with rheumatoid arthritis, rheumatic fever and systemic lupus erythrematosus were under chronic corticosteroid therapy. Twenty-seven patients with various nonrheumatic diseases (cerebral vascular accident, congestive heart failure, various neurological syndromes) served as hospitalized, chronically ill, control subjects. These control subjects were comparable in age, although predominantly male (Table I). The patients with rheumatic diseases, as one might expect in rheumatoid arthritis and systemic lupus erythematosus, were predominantly female. Forty apparently normal subjects, the kindred of nonrheumatic patients, acted as normal ambulatory control subjects.

Each patient was inoculated subcutaneously with 0.5 ml of brucella vaccine (Parke-Davis) containing *Brucella abortus* and *melitensis*, 2,000 million killed bacteria per ml. In most cases, blood samples were obtained before inoculation and at 1, 2 and 3 weeks after inoculation. In 2 patients, samples were obtained at 2-day intervals. In 12 cases, samples were taken once a week for periods of months. In many instances the following agglutinations were performed on the several samples of a single patient simultaneously.

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Antibrucella agglutinins were measured by the slide agglutination technique, with diagnostic febrile antigen for brucella (Lederle), according to the method of Huddleson and Abell (6). The sera of 2 control patients and 2 rheumatic patients were independently studied by Dr. D. Widelock of the Department of Health, New York City, and comparable titers of brucella antibodies were found. Isohemagglutinin titers were determined as described by Kabat (7) and antiglobulin tests were performed as described by Rosenfield, Vogel and Rosenthal (8). Lupus erythematosus cell preparations were made in accordance with the method of Lee (9), and antistreptolysin titers were done by the method of Rantz and Randall (10).

Antinuclear antibody titers were performed by a fluorescent antibody technique described previously (11) with this major modification: imprints of mouse spleen, liver and kidney were used as a source of nuclei, rather than chicken red cells. Slides so prepared were fixed in acetone for 10 minutes and used immediately with the usual indirect staining method. The fluorescent conjugates were absorbed twice for 1 hour with guinea pig liver powder in order to remove nonspecific fluorescence.

Anti-thyroglobulin antibodies were detected in inactivated sera by means of a slide agglutination method, utilizing thyroglobulin-coated latex particles prepared by Hyland Laboratories. The test was performed in undiluted serum and at a dilution of 1 : 10 in order to allow for any prozone phenomenon. Whenever the diluted or undiluted serum was positive, serial dilutions were done to determine the exact titer. Forty control sera from blood bank donors were negative and 19 of 20 patients with suspected thyroid disease were negative. One patient with clinical Hashimoto's disease had a positive serum. The positive control serum provided by Hyland Laboratories and considered very strongly positive had a titer of 1 : 64. Latex particle fixation was done by the method of Singer and Plotz (12). Influenza antibodies type A_2, B Lee, PR8, FM, and SW were measured in 17 selected sera of 5 patients with systemic lupus erythematosus by Dr. D. Widelock of the Department of Health of New York City.

Titers of all antibodies will be expressed as the reciprocal of the dilution. All of the antinuclear antibodies, lupus erythematosus preparations, and anti-thyroglobulin antibodies were done on all of the samples of each patient at the same time. Serum complement titrations were determined with slight modifications according to the technique described by Kabat and Mayer (13). Paper electrophoresis was performed in a Durrum-type cell (Manual of Paper Electrophoresis, model R, Beckman Instrument Co., 1958). The absolute values of different fractions were determined as the percentage of total protein measured by the biuret method or by refractometry.

Sedimentation characteristics of the antibrucella agglutinins, the rheumatoid factor, and the antinuclear antibodies were studied in a Spinco model L ultracentrifuge by 2 different procedures. Initially, serum diluted in saline was centrifuged at 40,000 rpm in a 40.2 rotor for 1 to 2 hours; after centrifugation, serial aliquots of 8 successive layers were removed consecutively from the top to the bottom of the tube. Afterward, better results were obtained by means of the zone centrifugation technique. This was done with the swinging bucket SW 39 rotor, using a sucrose density gradient, as described by Kunkel (14).

TABLE I

Control patients

Patient	Diagnosis	Age	Sex	Antibrucella titer			
				Initial	1 wk	2 wks	3 wks
G.B.	As. ht. dis.	72	M	neg	320	640	80
A.B.	As. ht. dis.	64	M	neg	neg	neg	neg
S.T.	CVA*	78	M	neg	20	40	80
M.R.	Paget's	60	M	neg	neg	neg	
J.B.	Meningioma	50	M	neg	neg	neg	neg
E.C.	Bronchogenic ca.	64	M	neg	40	40	
B.	Metastatic brain ca.	67	M	neg	neg	20	40
De.C.	Bronchogenic ca.	50	M	neg	80	640	
D.G.	As. ht. dis.	58	M	neg	20	640	160
G.C.	Ca. of rectum	50	M	neg	160	640	160
M.L.	Pancreatitis	72	F	neg	neg	320	320
G.D.	Arteriosclerosis	69	M	neg	neg	neg	
J.M.	CVA	80	M	neg	40	160	80
J.M.	Arteriosclerosis	71	M	neg	neg	40	80
W.S.	Arteriosclerosis	64	M	neg	20	80	80
G.K.	CVA	70	M	neg	neg	80	20
J.S.	CVA	62	M	neg	40	80	160
J.T.	Hemangioma	39	M	neg	neg	80	160
A.M.	Syringomyelia	42	M	neg	160		320
V.J.	Congestive ht. dis.	40	F	neg	neg	160	
T.C.	Congestive ht. dis.	34	M	neg	80		
A.C.	Cirrhosis	60	F	neg	160		
F.H.	Rheumatic ht. dis.	44	F	neg	20	80	80
E.D.	Rheumatic ht. dis.	23	F	neg	80	80	40
T.H.	Pyelonephritis	32	F	neg	40		
M.H.	Psoriatic arthritis	39	F	neg	40		320
A.S.	Paralysis agitans	69	M	neg	40	80	40

* Cerebral vascular accident.

TABLE II

Antibrucella titers for all groups *

Group	Sample size	Geometric mean antibrucella titer		95% Confidence limits
		Initial	2 wks	
Family members	40	0	210	150– 300
SLE	10	0	400	240– 660
RA	13	0	555	350–1,000
Other diseases	17	0	80	40– 155

* SLE = systemic lupus erythematosus; RA = rheumatoid arthritis.

RESULTS

A. Brucella antibodies

Table I summarizes the antibrucella titers in the hospitalized control subjects at 7, 14 and 21 days. The average geometrical mean titer was 80 at the end of 2 weeks. A summary table of all

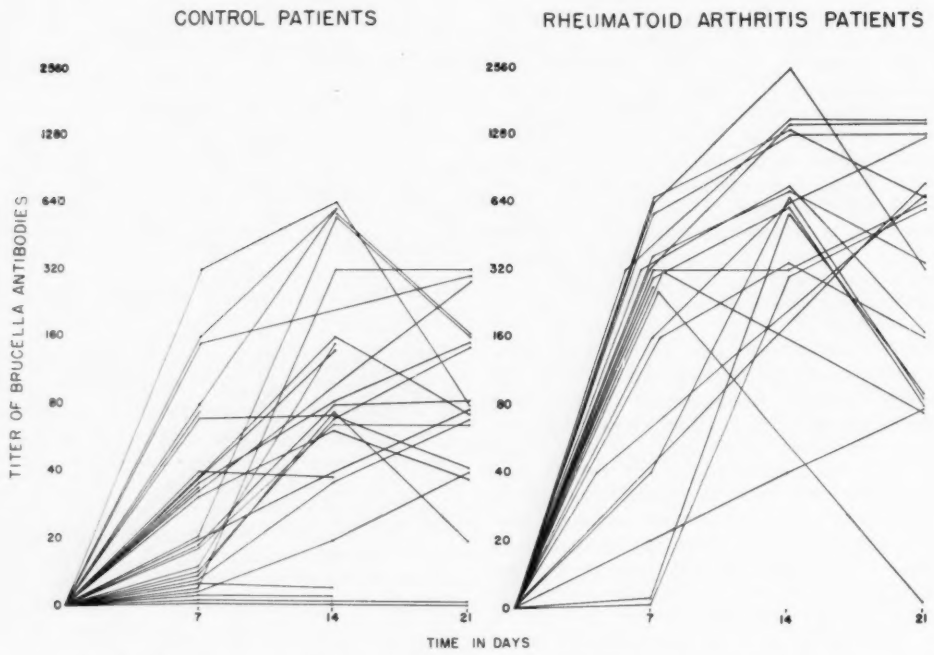

FIG. 1. ANTIBRUCELLA TITERS IN CONTROL SUBJECTS AND RHEUMATOID ARTHRITIS PATIENTS.

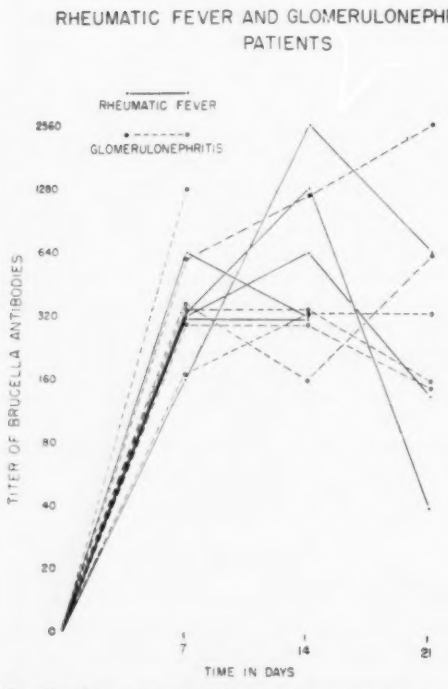

FIG. 2. ANTIBRUCELLA TITERS IN PATIENTS WITH RHEUMATIC FEVER AND GLOMERULONEPHRITIS.

groups at the 2-week interval shows that both the family control group and the hospitalized patient control group were significantly less reactive than the rheumatoid arthritis group [1] (Table II). Because the sample number in the systemic lupus erythematosus group was 10, the p value is not significant, but the difference between groups would be significant if the sample were larger.

Brucella antigen had been chosen for these experiments because it was presumed that it had not been met by the subjects and hence was satisfactory for stimulation. The possibility exists that for some of our patients the antigen was not unfamiliar. Of the total of 68 patients inoculated, three had positive antibrucella agglutinin titers before inoculation. These three, with titers of 20, 40, and 80 were in the systemic lupus erythematosus group. The mean titer at 2 weeks in the rheumatoid arthritis group was 555 and in the systemic lupus erythematosus group was 400. The small size of the rheumatic fever and glo-

[1] p Value for difference between systemic lupus erythematosus patients and normal family members = 0.075; p value for difference between rheumatoid arthritis patients and normal family members < 0.01.

TABLE III

Laboratory data on systemic lupus erythematosus patients

Patient	Antibrucella titers				LE cells	Antinuclear antibodies		Antiglobulin test		Isohemagglutinins		γ-Globulin		Latex particle fixation		Complement (50% units)		Anti-thyroglobulin antibodies	
	Initial	1 wk	2 wks	3 wks		Initial titer	2 wks	Initial	Later	Initial titer	2 wks	Initial	2 wks	Initial	2 wks	Initial	2 wks	Initial titer	2 wks
M.L.	20	20	1,280		neg	4	4	neg	pos. (2 wks)	B: 128	64	1.82	2.00	20	neg	61	48	neg	neg
J.H.	neg	640	1,280	1,280	neg*	16	32	neg	pos (4 wks)	A: 32 B: 64	128 128	1.31	1.05	neg	neg	76	60	neg	16
M.P.	neg	160	640	320	pos	32	16	neg	neg	B: 4	4	1.74	1.65	neg	neg			4	4
N.R.	neg	neg	80	neg	pos	256	128	neg	neg	A: 64 B: 64	32 16	2.52	2.49	neg	neg	100	70	4	32
R.W.	neg	320	640	320	pos	8	16	neg	pos (2 wks)	B: 64	32	1.00	0.94	neg	neg			neg	2
M.S.	40	160	320	1,280	pos	16	32	neg	neg	A: 128 B: 32	128 32	1.18	0.95	160	neg				neg
J.E.	neg	neg	160	40	pos	512	256	neg	neg	A: 64 B: 128	64 64	1.82	1.57	20	neg			neg	8
M.S.	80	80	320	320	pos	16	16	neg	pos.† (8 wks)	A: 16	8	1.18	1.34	neg	neg	52	55	neg	neg
M.M.	neg	320	320	320	pos	64	32	neg	neg	A: 64 B: 32	32 32	1.20	1.71	neg	neg	64	56		
J.R.	neg	320	1,280	640	pos	256		neg	neg			1.71		neg	neg				
T.A.	neg	20	320	320	pos	64	64	neg	neg	A: 32 B: 32	128 64	1.54	1.12	neg	neg			neg	neg

* Patient had positive preparations on previous examination.

† Positive Coombs occurred 4 weeks after polio immunization and 2 weeks after anamnestic brucella agglutinin rise from 160 to 640. Titers are expressed as the reciprocal of the dilution.

TABLE IV

Influenza antibodies *

Patient	Initial titer					Later†				
	A2	B Lee	PR8	FM	SW	A2	B Lee	PR8	FM	SW
M.P.	32	64	128	64	0	32	128	64	64	0
R.W.	32	0	0	32	0	32	0	0	16	0
M.M.	0	32	64	32	64	0	32	64	16	64
J.E.	0	0	0	64	0	0	0	0	512‡	0
M.S.	0	16	32	16	32	0	4	32	16	64

* Titers are expressed as the reciprocal of the dilution.
† These samples were taken at the time of maximal production of brucella agglutinins.
‡ This rise occurred 2 weeks after booster dose of brucella vaccine.

merulonephritis groups does not permit statistical evaluation, but these also showed high titers at 14 days (160 to 2,560; Figures 1 and 2). The three systemic lupus erythematosus patients who had initial positive titers showed in general no difference in response to brucella antigen inoculation from the other patients with this diagnosis. Comparison of the mean titers at Days 7, 14 and 21 showed that at the latter two days, patients in the rheumatic disease group had significantly higher titers than those in the control series, with values of less than 0.001 for rheumatoid arthritis. More frequent bleedings done on two patients showed that the peak response occurred between the eighth and tenth day after inoculation.

B. Effect of brucella antigen inoculation on production of other antibodies

Rheumatic fever and glomerulonephritis. No significant rise of antistreptolysin titer was noted in this group of patients.

Systemic lupus erythematosus (Table III). Isohemagglutinin titers, antinuclear antibodies, complement levels and total serum γ-globulin did not show appreciable change in these patients following brucella antigen stimulation. In 4 of the 11 patients in this group, however, transiently positive direct antiglobulin tests were observed during the peak immune response to brucella. In five patients with systemic lupus erythematosus, serial blood samples were examined for antibodies to various influenza viruses [2] (Table IV). Four of these patients showed patterns in their influenza antibodies which are not unusual for their age group in New York City; these patterns did not change during the immunization period. In one patient, however, there was an initial titer of 64 for the FM 1 strain. Two weeks after a subcutaneous injection of 0.1 ml of brucella antigen, this titer rose to 512, although titers to all of the other strains tested remained zero. It is possible that this patient had an infection with the FM 1 virus during this period, although this virus has not been isolated in New York City in recent years.

Anti-thyroglobulin antibodies were detected before immunization in two patients in titers of 4 and 64 (Table III). Two weeks after stimulation these antibodies were detected in three additional patients in titers of 2, 8 and 16, although the original two patients did not have any subsequent change in their titers.

Rheumatoid arthritis. Titers of isohemagglutinins and serum γ-globulin showed no significant change. One patient developed a positive Coombs test 8 weeks after brucella inoculation.

In seven patients of this group serial determinations of the latex particle fixation were made. In three of these seven there was significant parallel rise of latex particle fixation titer as the brucella agglutinin titer rose (Figure 3).

Since it is well known that the disulfide bridge of the macroglobulins can be disrupted by the addition of sulfhydryl-containing compounds with subsequent loss of antibody activity, mercaptoethanol was added to seven high-titer brucella and latex sera in 0.01 M concentration. There resulted a marked loss of agglutinating activity of both the brucella and latex system. Ultracentrifugation of these sera revealed that both the brucella agglutinins and the latex particle fixation activity resided in the 19S component and that the 7S component was inactive in both systems. Similarly, the brucella agglutinins resided in the 19S fraction of the control sera (Tables V and VI).

In order to differentiate clearly both 19S activities, the same seven test sera (with both a high titer of brucella antibodies and a high titer in the latex particle fixation) were repeatedly absorbed with brucella antigen until no brucella antibodies could be demonstrated. After absorption these sera still had their initial activity in the latex particle fixation system.

Before stimulation with brucella antigen no

[2] Courtesy of Dr. Daniel Widelock, Bureau of Laboratories, Department of Health, New York City.

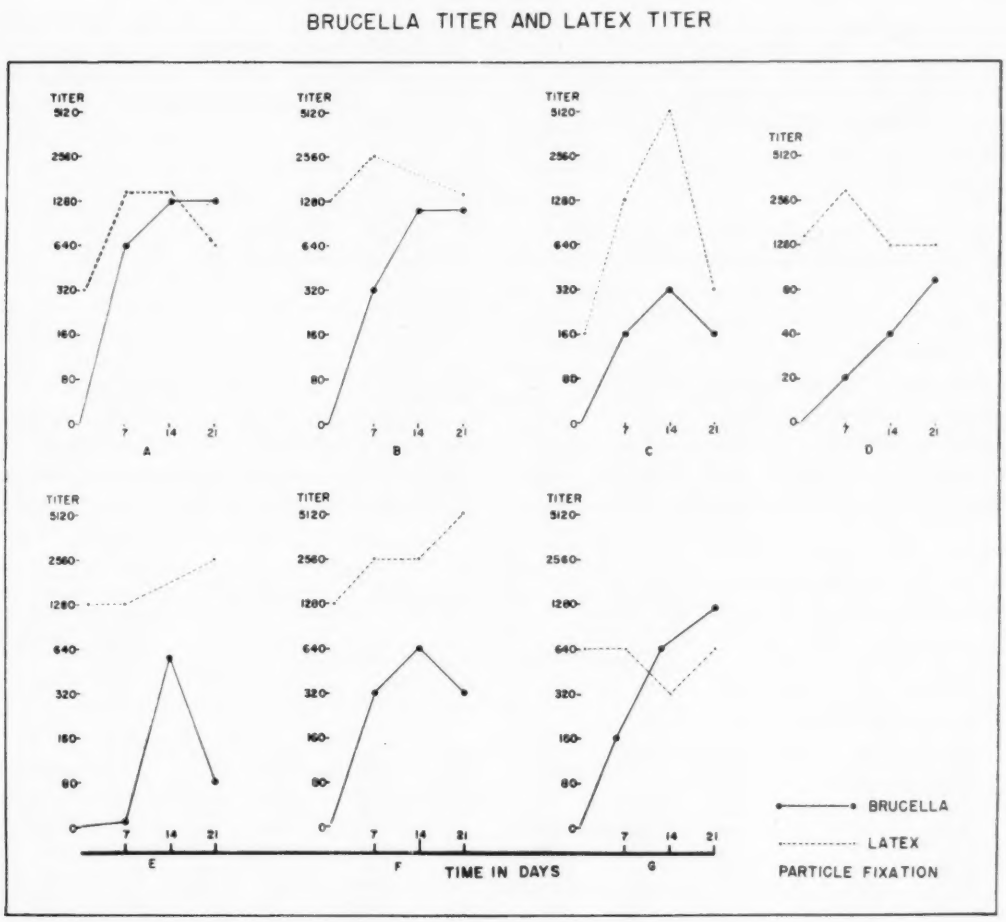

FIG. 3. BRUCELLA AND LATEX TITERS IN PATIENTS WITH RHEUMATOID ARTHRITIS.

patient with rheumatoid arthritis, of 15 tested, had detectable anti-thyroglobulin antibody. Approximately 2 weeks after inoculation these antibodies were detected in five patients in titers of 1, 1, 32, 2 and 2 (Table VII).

Control group. No latex particle fixation activity or lupus erythematosus cell factor was found in the initial serum of any of the control group. In the initial sera of 16 patients, no anti-thyroglobulin antibody was noted. In 13 of these patients no anti-thyroglobulin activity was found after inoculation with brucella antigen and during the period of brucella agglutinin response; γ-globulin in the control patients did not vary in the serial samples.

DISCUSSION

In this series of experiments, there were statistical differences in antibody response between the rheumatic disease patients and the control patients after brucella stimulation. The agglutination reaction used to detect the antibrucella agglutinins

TABLE V

Brucella antibody titer

		Serum alone	Serum + mercaptoethanol (0.01)
R.W.	SLE	1:320	(−)
M.V.	RA	1:640	1:40
A.P.	RA	1:320	1:20
W.A.	RA	1:1,280	1:20

TABLE VI

Ultracentrifugation on sucrose gradient; latex and brucella titer of serum fractions

Serum alone			Serum+mercaptoethanol		
Layer	Latex	Brucella	Layer	Latex	Brucella
1	(−)	(−)	1	(−)	(−)
2	(−)	(−)	2	(−)	(−)
3	(−)	(−)	3	(−)	(−)
4	(−)	(−)	4	(−)	(−)
5	(−)	(−)	5	(−)	(−)
Pellet(+)	1:3,200	(+)1:1,600	Pellet(+)	1:200	(−)

A.P. (RA) Original serum:
latex 1:2,560; brucella 1:640

was specific. Parallel agglutinations using *Salmonella typhosa* were uniformly negative. Antibrucella agglutinins in systemic lupus erythematosus patients were distinct from antinuclear antibodies, and in rheumatoid arthritis patients were demonstrated to be separate from rheumatoid factor. Further, rheumatoid factor had no enhancing effect when added to low-titer antibrucella sera.

The possibility that the agglutinating phenomenon was related nonspecifically to the presence of

TABLE VII

Antibody titers of rheumatic patients and patients with acute glomerular nephritis

			Antibrucella titers				Latex particle fixation titer		LE prep.	Anti-thyroglobulin titers	
Patient	Age	Sex	Init.	1 wk	2 wks	8 wks	Init.	2 wks		Init.	2 wks
Rheumatoid arthritis											
A.P.	19	F	neg	640	2,560	320	neg	neg	neg		
A.P.	35	F	neg	640	1,280	1,280	320	1,280	neg		neg
R.B.	65	F	neg	160	640	1,280	640	640	neg	neg	neg
E.G.	40	F	neg	320	320	640	neg	neg	neg	neg	neg
H.S.	40	M	neg	320	640		320	80	neg	neg	neg
I.R.	33	F	neg	320	640	80	neg	neg	neg		neg
M.G.	32	F	neg	640	1,280	640	40	20	neg	neg	1
W.A.	51	M	neg	320	1,280	1,280	1,280	2,560	neg	neg	neg
A.A.	48	F	neg	160	320	160	160	5,120	neg	neg	32
M.Q.	62	F	neg	40		640	2,560	2,560	neg	neg	
A.A.	30	M	neg	320		neg	neg	neg	neg	neg	2
L.N.	66	F	neg	320		1,280	neg	neg	neg	neg	2
P.S.	62	M	neg	20	40	80	1,280	2,560	neg	neg	neg
A.D.	45	F	neg	40	640	80	neg	neg	neg	neg	neg
R.C.	61	M	neg	320	640	320	1,280	2,560	neg		
P.W.	60	F	neg	320	640	160	neg	neg	neg	neg	neg
M.P.	60	F	neg	320		80	neg	neg	neg	neg	
D.R.	70	F	neg			320	neg	neg	neg	neg	
R.A.	64	F	neg	neg	640	80	1,280	2,560	neg	neg	1
Acute rheumatic fever											
F.W.	6	F	neg	640	1,280	320					
M.V.	17	M	neg	160	2,560	640					
P.B.	7	F	neg	320	640	40					
J.B.	11	M	neg	320	320						
G.R.	10	F	neg	320	1,280	40					
Acute glomerular nephritis											
S.H.	13	M	neg	40	320	160					
J.K.	9	F	neg	160	320	160					
T.M.	42	F	neg	640	1,280	2,560					
M.S.	12	M	neg	80	320	160					
M.W.	8	M	neg	1,280							
J.S.	22	M	neg	320	160	640					

complement was discarded, because in six instances simultaneous measurements of complement and antibrucella agglutinins revealed negligible complement activity in the presence of high agglutinating activity.

The present study confirms the suggestion that patients with rheumatic diseases may hyper-react to brucella antigen as previously reported by Rejholec (2). The data are in disagreement with the results obtained by other groups who have used diphtheria toxoid (5), pneumococcal polysaccharide (4) or typhoid vaccine (3). Resolution of this difference seems possible only on the basis that in the same individual there are different quantitative responses to various antigens. Brucella seems to be one antigen which is capable of eliciting a high antibody response in rheumatic disease patients. The known propensity for the brucella organism to produce tissue damage in mesenchymal structures, the target tissue in rheumatic diseases, possibly suggests a basis for understanding the present results.

What specific antigens in the brucella organisms are responsible for this hyper-response, and what other antigens share this property, are questions which are still unanswered. In the present series, patients with four distinct syndromes shared this capacity for developing antibody in high titer to brucella antigen.

Among the systemic lupus erythematosus patients, three had measurable antibrucella titers even before inoculation of the test brucella antigen. One of these patients was born in Malta, and may have had brucella infection. Its occurrence in the other two systemic lupus erythematosus patients suggests that perhaps the elevated baseline titer represents in them another abnormal response in antibody formation among the many which characterize this disease.

Of all the other antibodies studied, only four appeared to increase in titer secondarily to stimulation by brucella antigen. Four of 11 patients with systemic lupus erythematosus showed transiently positive direct *antiglobulin* (Coombs) tests; 1 of 5 systemic lupus erythematosus patients showed a marked rise in titer of an unusual *influenza antibody* (FM 1); and 3 of 7 rheumatoid arthritis patients showed possible (and 3 others showed suggestive) rises in *latex particle fixation titer,* admittedly a crude measure of rheumatoid factor. *Anti-thyroglobulin* antibodies occurred after brucella inoculation in 3 of 11 patients with systemic lupus erythematosus, and in 5 of 15 patients with rheumatoid arthritis. In all these instances the antibody activities measured were actually separate from the brucella agglutinins.

In all instances, when positive, the Coombs test became positive 2 to 8 weeks after stimulation; in two instances, the presence of an anti-red cell factor paralleled a sudden increase in titer of brucella agglutinins. The very high titer against influenza antibody occurred 2 weeks after a second dose of 0.1 ml of brucella vaccine.

Detectable anti-thyroglobulin antibody occurred in titers of 16, 2 and 8 in the systemic lupus erythematosus patients after inoculation. In two of the patients (J.H. and R.W.) anti-red cell antibody was transiently present at the same time. In the third patient (J.E.) the unusual titer to FM 1 strain of influenza virus, previously alluded to, had occurred. Two systemic lupus erythematosus patients had anti-thyroglobulin antibody before brucella inoculation; these showed no increase in their titer of anti-thyroglobulin antibody.

Prior to stimulation, no patient with rheumatoid arthritis had detectable anti-thyroglobulin antibody; 2 weeks later, 5 of 15 patients tested had detectable antibody. One of these 5 patients developed a titer of 32 at the same time that she developed an increase in the latex particle fixation titer from 160 to 5,120.

Thus, of a total of 11 antibody activities studied in rheumatic disease patients after brucella inoculation, significant alterations (appearance *de novo* or definite increase of titer) were observed in 4. These alterations occurred in 7 of 19 patients with rheumatoid arthritis and in 5 of 11 patients with systemic lupus erythematosus. Among the rheumatic disease subjects exhibiting this type of reactivity, there was a tendency for multiple changes in individual patients. Four patients with systemic lupus erythematosus and one with rheumatoid arthritis showed alterations in two different antibody activities (anti-red cell plus anti-thyroglobulin in three patients, anti-red cell plus anti-influenza in one patient, and rheumatoid factor plus anti-thyroglobulin in one).

The significance of this broad spectrum of antibody response to a single antigenic stimulus is

obscure. All rheumatic disease subjects did not show this phenomenon, but it seems likely that if more antibody activities had been measured, more reactive patients would have been found. It is possible, too, that the pattern which appears here—single antigenic stimulus yielding broad scattering of antibody response—may provide a basis for understanding the wide range of abnormal antibody activities found in systemic lupus erythematosus and rheumatoid arthritis. Autoimmunity has been thought to require some alteration of a normal tissue component to create antigenicity of this tissue (15) or the sudden availability of a normal tissue component for the immune system (16). The present results could be interpreted as indicating that the altered host response may be a basic defect in some "autoimmune" diseases.

There is some support for the hypothesis that this altered immune response may be genetically determined. This possibility has been suggested by the findings of serological and clinical abnormalities in the kindred of patients with systemic lupus erythematosus and rheumatoid arthritis (17–20).

In these studies no control patient showed response to brucella inoculation in any antibody activity other than that directed specifically against brucella; this lends support to the suggested hypothesis of an altered immune response. Hackett and Beech (21), however, have recently reported that during the course of active immunization with heterologous antigens in seven presumably healthy young women, two of these, with previously demonstrated anti-thyroglobulin antibodies, developed complement-fixing antibodies to liver and kidney tissue. Further studies of this sort with larger numbers of subjects may help to clarify this issue.

Seven of the "antibody" activities studied failed to participate in the "nonspecific" response. Attempts to analyze the two groups of antibodies defined by this response have not yet been carried very far. Antibodies have been classified in many ways: chiefly either according to the biological or physical nature of the antigen, or according to the physicochemical characteristics of the antibody. The 11 antibodies studied contained activities against isologous, heterologous and autologous antigens. The four "responding" antibodies do not segregate according to this classification; they include three autologous and one heterologous. Antigens may be classified as being particulate or soluble. This classification also does not serve to single out the "responding" group of antibodies.

Antibodies may be classified by their electrophoretic and sedimentation characteristics into three groups: γ-2 (7S), γ-1-M (or β-2-M) (19S), and β-2-A. Attempts are now being made to define the antibodies studied according to this classification. The antibrucella agglutinins in the present series have been shown to belong to the γ-1-M group, as does the rheumatoid factor. It seems possible that this approach may be a fruitful one and that all the "responding" antibodies may belong to this class. Studies are now under way to stimulate antibodies of another class in similar patients.

SUMMARY

1. Forty-one patients with various rheumatic diseases and 27 control patients were inoculated with brucella vaccine. As a group the patients exhibited a significantly greater rise in antibrucella agglutinins compared with the controls. Some overlap in both groups was present.

2. Alterations were noted in other antibody systems—anti-red cell (Coombs), anti-thyroglobulin, and possibly in the influenza antibody and rheumatoid factor—after this primary stimulation in some of the patients with rheumatic diseases, but no titers for these antibodies were noted in the control patients.

3. The effect of brucella antigen in these patients may be related to the damage that this organism can produce on mesenchymal tissue.

ACKNOWLEDGMENT

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STUDIES ON STARLING'S LAW OF THE HEART. V. LEFT VENTRICULAR FUNCTION IN MAN

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There is general agreement that the force of contraction developed by strips of cardiac muscle is a function of the length of the fibers at the onset of contraction (1–3). Similarly, in the isolated heart (4), the heart-lung preparation (5) and in the open-chest, anesthetized dog (6–8), the energy of contraction, as reflected by the external work performed by the ventricle, has been shown to be dependent upon the ventricular end-diastolic fiber length or pressure. These observations have been interpreted to be consistent with the concept enunciated by Starling in his Linacre lecture on "The Law of the Heart (9).

However, no such general agreement exists regarding the applicability of Starling's law of the heart to the circulation of human subjects. This lack of information regarding the relationship between left ventricular end-diastolic pressure (and fiber length) and the characteristics of ventricular contraction in man is understandable when it is considered that only recently have methods been developed which are suitable for the direct measurement of left ventricular pressure in subjects without cardiovascular disease (10). One of the experimental approaches which has been employed to test the validity of Starling's law has been a study of the hemodynamic response resulting from acutely induced hypervolemia (11–15). A variety of fluids has been infused in attempts to simulate in man the increase in venous return to the Starling heart-lung preparation. In several such investigations an augmentation of the total blood volume has not been associated with a significant elevation of cardiac output (13–15).

The purpose of the present investigation was to define the relationships between left ventricular end-diastolic pressure and the characteristics of left ventricular contraction in a group of subjects without cardiovascular disease. Acutely induced hypervolemia was combined with continuous ganglionic blockade in order to produce significant variations in ventricular filling and thus to permit an examination of Starling's law of the heart.

METHOD

Six male subjects averaging 25 years in age were studied. All had provided informed consent for these studies. Five of these subjects were referred to the National Heart Institute for cardiac catheterization because of the presence of systolic heart murmurs. Clinical examination was otherwise negative, chest roentgenograms and electrocardiograms were within normal limits, and these murmurs were considered to be functional in origin. The sixth subject, J.M., a 32 year old male, was studied three months after repair of a small ventricular septal defect. He had never experienced symptoms of diminished cardiac reserve, had not had cardiac enlargement on roentgenographic examination and his pulmonary arterial pressure was within normal limits (16) prior to operation as well as at the time of this study. Three or four phlebotomies were performed on each subject over a period of 7 to 10 days and the 1,500 ml of blood so obtained was stored in solutions of sodium citrate, citric acid and dextrose. The studies were carried out 2 to 4 days after the last phlebotomy; the subjects were in a basal, postabsorptive state and had received 100 mg of pentobarbital orally 30 minutes before their arrival in the laboratory. Local anesthesia was applied to the pharynx by means of a Pontocaine (tetracaine) spray; a small rubber balloon of the type described by Schilder, Hyatt and Fry (17) was attached to a plastic catheter and introduced into the midesophagus. The right saphenous vein was then exposed and a right heart catheterization was carried out. The pressures in the right side of the heart and pulmonary artery were within normal limits (16) in all six subjects and an inhaled radioactive krypton test (18) carried out with the catheter tip in the pulmonary artery confirmed the clinical impression that a left-to-right shunt was not present. It was then decided to proceed with the remainder of the study; the catheter employed for the right heart catheterization was removed and transseptal left heart catheterization was carried out in a manner described in detail elsewhere (10). After introduction of the transseptal needle into the left atrium, a polyethylene catheter was passed through this needle into the left ventricle.

A 75 cm polyethylene catheter (PE no. 50) was introduced through an antecubital vein by means of percutaneous puncture, and was advanced into the superior

vena cava or right atrium. The ganglionic blockade was then induced by an intravenous infusion of a thiophanium derivative, Arfonad (trimethaphan), a drug which also has some direct vasodilatory action (19, 20). The trimethaphan was administered into a peripheral vein by means of an infusion pump at a rate which was constant for each subject and which ranged from 0.6 to 3.8 mg per minute, with an average rate of 1.7 mg per minute; it was continued at this rate for the entire study. These infusion rates lowered systolic arterial pressure by 31 to 51 mm Hg, with an average decline of 42 mm Hg (Table I). The effectiveness of the reduction of reactivity of the vascular system which had been achieved by the trimethaphan was tested by means of the cold pressor test; the response of systemic arterial pressure to the immersion of a foot into ice water for 1 minute was examined both before and after the infusion of trimethaphan had been begun. In all instances the elevation in arterial pressure which occurred before the trimethaphan infusion was eliminated when the cold pressor test was repeated during the trimethaphan infusion.

Cardiac output and pressures in the brachial artery, left ventricle and esophagus were then measured. Then the 1,500 ml blood, which had been warmed to body temperature, was infused during a period of 30 to 50 minutes. Cardiac output and pressure measurements were carried out at intervals of approximately 10 to 15 minutes during the infusion. In five of the subjects a total of four measurements and in one subject three measurements of cardiac output and pressures were made.

Brachial arterial pressure was measured through an indwelling needle by means of a Statham P23A pressure transducer. A model P23D transducer was employed for the measurement of left ventricular pressure. A Statham transducer, model P.M.131T, was utilized for the measurement of intraesophageal pressure (17). Cardiac output was determined by the indicator dilution technic and was calculated in the manner described by Hamilton, Moore, Kinsman and Spurling (21). Indocyanine green dye was injected into the vena cava or right atrium via the polyethylene catheter. Arterial blood was sampled through the indwelling arterial needle and pulled through a cuvet densitometer (22) by a motor-driven syringe at a rate of 20 ml per minute. The response of the densitometer to an instaneous change in density is 95 per cent in 1 second (23). The validity of these technics employed in the measurement of the cardiac output had first been demonstrated by a comparison with flowmeter measurements in the open-chest dog (24). In another study, utilizing identical technics, cardiac output was measured in duplicate on 20 occasions. The standard deviation of the differences between these determinations was 182 ml per minute or 6 per cent of the mean value of the cardiac output (25).

Minute work of the left ventricle in kilogram-meters per minute was calculated as the product of mean arterial pressure in centimeters of H_2O, and the cardiac output in liters per minute divided by 1,000. Left ventricular stroke work in kilogram-centimeters was calculated as the product of mean arterial pressure in centimeters of H_2O and the stroke volume in milliliters divided by 100. The tension-time index in millimeters Hg × seconds was calculated from the area under the systolic portion of the arterial pressure pulse, as described previously (26). Left ventricular power in watts was calculated in five subjects by dividing left ventricular stroke work by the duration of systole. The conversion factor utilized was: 1 watt = 10,180 g-cm per second. The duration of systole was calculated as the time interval between the onset of the isometric contraction period of the left ventricle and the time of aortic valve closure; the latter was determined by noting the instant at which the left ventricular pressure pulse reached the level of the dicrotic notch of the brachial arterial pressure pulse. The mean rate of left ventricular ejection could be determined in three subjects; it was calculated by dividing the stroke volume by the duration of ejection. In some tracings, artifacts in the pressure pulses prevented accurate estimation of these time intervals. Left ventricular end-diastolic pressure was determined as the difference between left ventricular

TABLE I

Blood pressure and heart rate before and after trimethaphan administration

Subject	Hematocrit	Control BAP	Pretransfusion		Post-transfusion	
			BAP	HR	BAP	HR
	%	*mm Hg*	*mm Hg*		*mm Hg*	
J.Ma.	35.2	134/80	101/69	91	95/59	86
J.M.	34.5	125/71	77/48	112	115/81	106
D.B.	28.7	138/70	107/67	88	105/89	103
B.E.	35.0	144/64	93/53	86	108/70	92
M.R.	35.0	145/82	104/60	107	121/73	110
J.B.	38.0	131/69	80/47	76	122/71	100
Average	34.8	136/73	94/57	93	111/71	99

* The control BAP refers to the brachial arterial pressure (systolic/diastolic) which existed at the onset of the study, after the placement of catheters but before the infusion of trimethaphan was begun. The BAP and HR (heart rate per minute) determined simultaneously with the first dilution curve, i.e., after stabilization of the circulation on trimethaphan but before the blood transfusion, are listed under "pretransfusion." Measurements under "post-transfusion" refer to observations made simultaneously with the final dilution curve, at the time of completion of blood transfusion.

and intraesophageal pressure at the end of diastole. All values of pressure and of time intervals reported were the average of the beats occurring during two respiratory cycles. The mean arterial pressure was obtained by electrical integration.

RESULTS

All subjects were mildly anemic as a result of the repeated phlebotomies. Their hematocrits at the time of study ranged from 34.5 to 38.0 per cent, except for D.B. in whom it was 28.7 per cent. At the time that cardiac output was measured for the first time, that is, just before transfusion, the brachial arterial pressure had been depressed by trimethaphan and ranged between 77/48 and 107/67, with an average level of 94/67 mm Hg. In spite of the hypotension, these subjects did not present the clinical appearance gen-

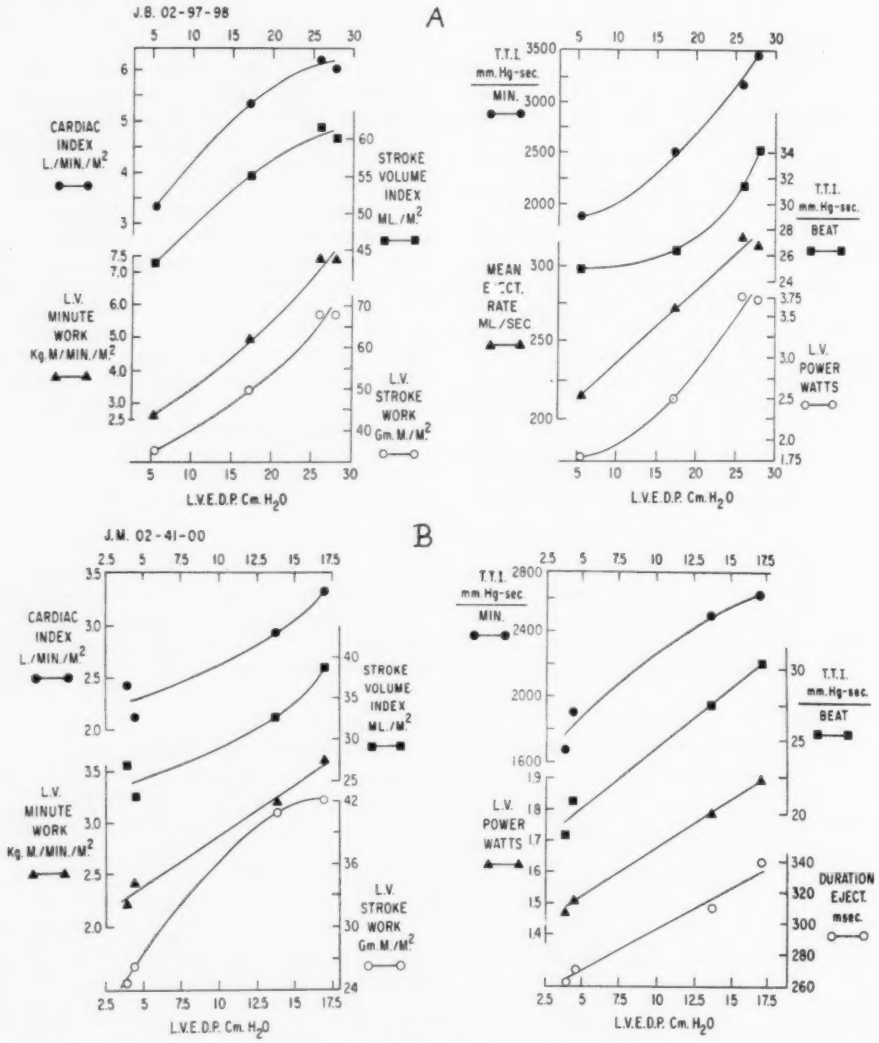

FIG. 1. RELATIONSHIPS BETWEEN L.V.E.D.P. (DIFFERENCE BETWEEN LEFT VENTRICULAR AND INTRAESOPHAGEAL PRESSURE AT THE END OF DIASTOLE) AND VARIOUS PARAMETERS OF LEFT VENTRICULAR (L.V.) PERFORMANCE IN SIX SUBJECTS. T.T.I. refers to the tension-time index; EJECT. refers to ejection.

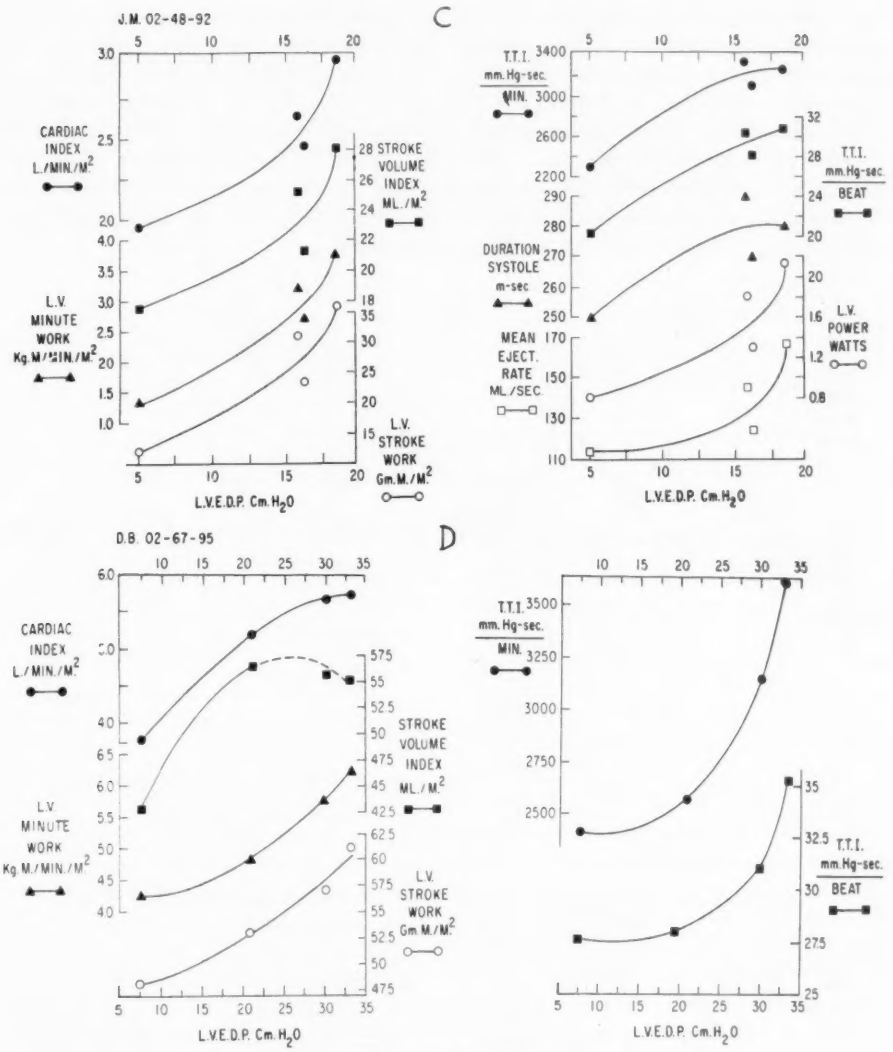

FIG. 1.—*Continued*

erally associated with shock; before transfusion the cardiac index exceeded 2.0 L per minute per m^2 in all subjects and averaged 3.15 L per minute per m^2.

As a result of the transfusion the arterial pressure rose significantly in four of the subjects, declined in one (J.Ma) and showed little change in one (D.B.). Heart rate changed only slightly in four subjects and increased significantly in two (D.B. and J.B.) (Table I). The results obtained in each subject are shown in Figure 1.

The left ventricular end-diastolic pressure at the onset of the blood infusion ranged from 1.2 to 7.6 cm H_2O and the average level was 4.8 cm H_2O. A significant elevation of left ventricular end-diastolic pressure occurred; at the completion of the infusion these pressures ranged from 17.0 to 33.1 with an average level of 22.2 cm H_2O. There were only slight alterations of intraesophageal pressure during the course of the blood infusions. The maximal change of this pressure in any subject was 3.0 cm H_2O and the reported

changes of "effective" end-diastolic pressure resulted primarily from alterations of the recorded intraventricular pressure. Representative pressure tracings are reproduced in Figure 2. In every instance the cardiac output rose along with the left ventricular end-diastolic pressure. The left ventricular work per minute and the left ventricular stroke work, the tension-time index per minute and per beat, the left ventricular power, duration of ejection and mean rate of left ventricular ejection also rose as the transfusion continued and as the effective left ventricular filling pressure rose (Figure 1). In five subjects stroke volume became elevated as left ventricular diastolic pressure rose. In the sixth subject (Figure 1 F) the stroke volume rose substantially at the beginning of the infusion, but declined slightly as it was continued.

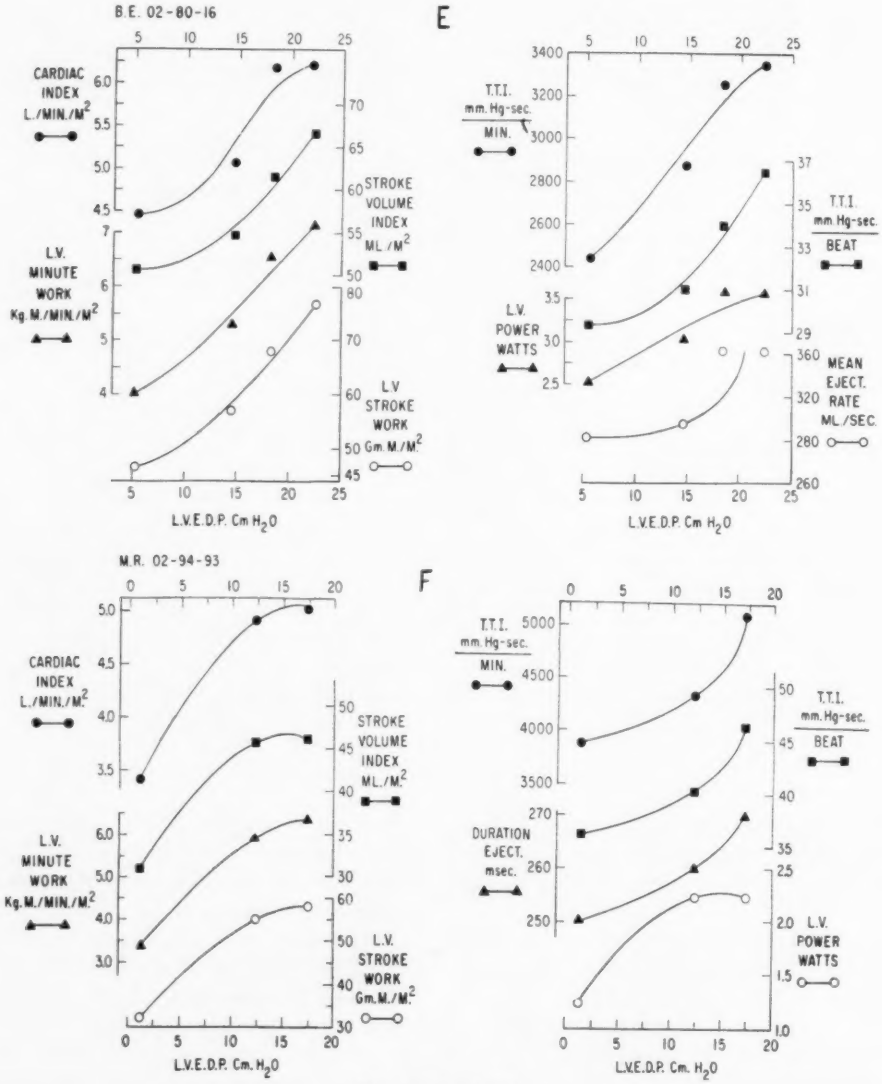

FIG. 1.—*Continued*

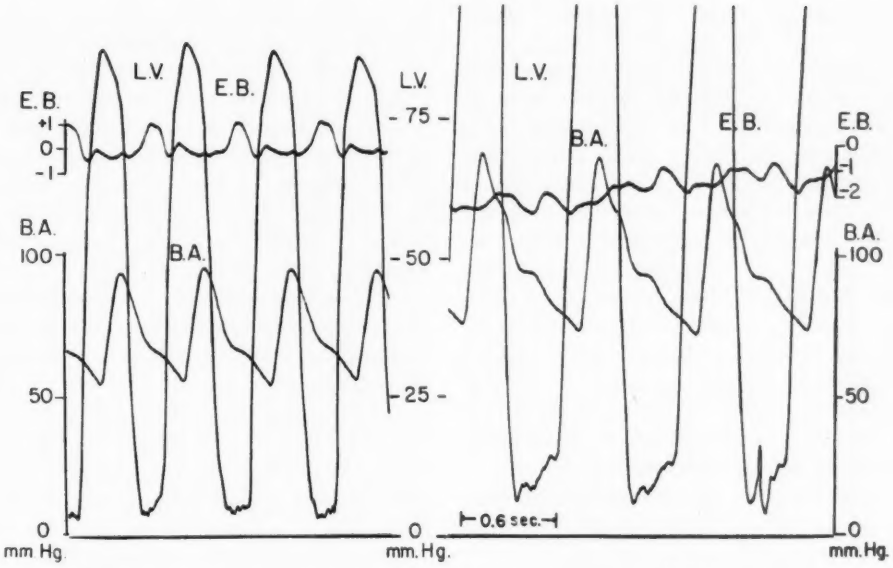

FIG. 2. SIMULTANEOUS PRESSURES RECORDED FROM THE LEFT VENTRICLE (L.V.), BRACHIAL ARTERY (B.A.) AND ESOPHAGUS (E.B.) BEFORE TRANSFUSION (LEFT) AND DURING TRANSFUSION (RIGHT).

DISCUSSION

It is clear from the studies of Sarnoff and his collaborators, carried out on open-chest, anesthetized dogs, that humoral (8) and neurogenic (27, 28) influences are capable of profoundly altering myocardial contractility, i.e., of elevating or depressing the ventricular function curve. Chapman, Baker and Mitchell have shown that the Frank-Starling principle applies to the closed-chest, anesthetized dog at rest and that during mild exercise the left ventricle operates at different "functional levels," i.e., with elevated ventricular function curves (29). Observations in this laboratory have demonstrated that norepinephrine is capable of markedly elevating the ventricular function curve in the closed-chest, unanesthetized dog (30). As has recently been indicated by Chapman (31), it would appear likely that humoral and neurogenic factors also exert similar influences in the intact human subject and that these factors may sometimes obscure the underlying relationship between ventricular end-diastolic pressure and the force of ventricular contraction. The purpose of the present investigation was not to assess the relative importance of these neurohumoral influences and of the Frank-Starling mechanism in the control of cardiac function in the intact organism; rather, it was designed to determine whether the Frank-Starling mechanism applies to the human heart when alterations of the neurohumoral factors are reduced to a minimum.

In order to accomplish this purpose, it would appear essential to relate ventricular end-diastolic fiber length or end-diastolic volume to the characteristics of ventricular contraction. Recent observations in the dog have demonstrated that the relationship between effective left ventricular end-diastolic pressure and segment length is unaffected by stimulation of the cardiac sympathetic nerves or of the vagi (32) and by large changes of systemic arterial pressure and of cardiac output (33). It has also been shown in man that in the absence of tachycardia, changes in the effective left ventricular end-diastolic pressure may be utilized to indicate alterations in left ventricular dimensions (34). Thus, without the availability of a suitable technic capable of providing accurate serial measurements of left ventricular volume in man, left ventricular and intraesophageal pressures were measured in order to permit measurement of changes in effective left ventricular end-diastolic pressure.

It is well recognized that when the intraesopha-

geal pressure is determined with the subject in the supine position, it does not provide an accurate measurement of the absolute level of intrapleural pressure (35, 36). However, as is evident in Figure 3 of the paper by Mead and Gaensler (35), even in this position, changes in the intraesophageal pressure satisfactorily reflect alterations in the intrapleural pressure. Accordingly, the difference between the left ventricular end-diastolic pressure and the intraesophageal pressure was measured during this study. Although no claim is made that the absolute effective end-diastolic pressure was provided by these measurements, it is felt that the magnitude of changes in effective left ventricular end-diastolic pressure which occurred as a result of the infusion could be determined with accuracy in this manner.

The importance of reflexes mediated through the autonomic nervous system in the control of myocardial contractility (27), as well as of the tone of the venous (37, 38) and arteriolar (39) beds is now well established. Rapid expansion of the blood volume could be expected to stimulate baroreceptors in the carotid sinuses, the aortic arch, the heart and lungs, as well as in the venous beds. As a consequence, it would be anticipated that a decline in ventricular and atrial contractility (27), bradycardia, venodilatation (37) and arteriolar dilatation (39) would occur. In this manner, reflex depression of the circulation could be expected to limit any augmentation of cardiac performance which might result from an increase in left ventricular end-diastolic fiber length, if the Frank-Starling mechanism were operative. In support of this hypothesis, it has been shown that the infusion of 1,500 ml of blood into intact subjects did not result in an augmentation of the central blood volume, of cardiac output and of cardiac work. It was postulated that the transfusion led to reflex venodilatation which, in turn, resulted in a tendency for the infused blood to pool in the venous system and thereby to diminish the volume of blood in the heart and lungs. However, when in the same subjects the activity of the autonomic nervous system was reduced by means of a ganglionic blocking agent, the transfusion of 1,500 ml of blood resulted in a striking augmentation of central blood volume and of cardiac output and work (15). Accordingly, with these observations in mind, it was considered that in order to ascertain whether the left ventricular end-diastolic pressure is a fundamental determinant of left ventricular performance, the blood should be infused after inhibition of the autonomic nervous system. The present investigation was therefore performed with the subjects receiving a continuous infusion of trimethaphan. The dose of this drug was sufficient to lower the arterial pressure significantly and to abolish the elevation of arterial pressure which normally occurs during the cold pressor test (40). It was thought that these hemodynamic alterations were produced by the ganglionic blocking activities of the drug (19, 40). Since there was no evidence of coronary artery disease in any of the subjects studied, it is considered unlikely that the moderate hypotension induced by the trimethaphan interfered sufficiently with coronary perfusion to depress cardiac function. This view is also supported by the observation that electrocardiographic alterations associated with myocardial ischemia did not occur.

In all six subjects studied the blood transfusion resulted in a striking elevation of left ventricular end-diastolic pressure. The magnitude of this increase in pressure is presumably related to the large increase in ventricular diastolic volume which is believed to have accompanied the transfusion. This was accompanied by an augmentation of left ventricular performance; the latter was evidenced by the increases which occurred in the cardiac output per minute and per stroke, the left ventricular work per minute and per stroke, the power (work per systolic second) of the left ventricle and the mean rate of left ventricular ejection (Figure 1). In addition, the tension-time index per minute and per beat also rose along with left ventricular end-diastolic pressure. The tension-time index has been shown in previous studies (26) to be the hemodynamic parameter which correlates most closely with myocardial oxygen consumption. These results then are in accord with the concept that Starling's law of the heart applies to the human subject.

An abbreviation of left ventricular systole generally accompanies the augmentation of cardiac performance which results from sympathetic stimulation (28); in these experiments, however, the duration of left ventricular systole increased (Figure 1; B, C and F). These results are not unexpected, since the observations were carried out

during ganglionic blockade and are compatible with the view that the hemodynamic changes observed reflected properties inherent in the cardiac muscle.

It was demonstrated in earlier studies carried out in this laboratory that marked alterations in left ventricular end-diastolic segment length and pressure occur as a consequence of variations in the duration of filling in the presence of atrial fibrillation (34) or as a result of variations in the effectiveness of atrial contraction in atrioventricular dissociation (41); the force of left ventricular contraction was found, in each instance, to be a function of the left ventricular end-diastolic segment length and, in the absence of tachycardia, of the left ventricular end-diastolic pressure as well. These results are entirely in accord with those obtained in the present investigation in which a different technic for varying ventricular filling was utilized. Evidence has also been presented recently that the force of atrial contraction in man is a function of the atrial pressure existing at the onset of atrial contraction (41), suggesting that the atria as well as the ventricles behave in accordance with Starling's law of the heart.

SUMMARY

This investigation was designed to determine whether Starling's law of the heart is applicable to man. Changes in effective left ventricular end-diastolic pressure were determined by measuring left ventricular pressure with a catheter introduced through the atrial septum, and intraesophageal pressure with a balloon. The activity of the autonomic nervous system was reduced with an infusion of Arfonad (trimethaphan), which was continued at a constant rate throughout the study. The reduction in circulatory reactivity produced by this drug was indicated by the absence of an arterial pressure elevation during the cold pressor test. Measurements of cardiac output, stroke volume, left ventricular work, power, tension-time index, and of the duration and mean rate of left ventricular ejection were carried out before, during the course of, and upon completion of a transfusion of 1,500 ml of the subject's own blood. This transfusion resulted in a significant elevation of left ventricular end-diastolic pressure in each subject, the average level rising from 4.8 to 22.2 cm H_2O. In each subject, as the left ventricular end-diastolic pressure rose, left ventricular performance, as reflected in the parameters listed above, also became augmented. These data are consistent with the hypothesis that under the conditions of these experiments the end-diastolic pressure is an important determinant of the characteristics of ventricular contraction and that Starling's law of the heart is applicable to man.

ACKNOWLEDGMENT

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EXPERIMENTAL REACTIVATION OF SUBSIDING RHEUMATIC FEVER

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When anti-inflammatory therapy is reduced or discontinued in patients with acute rheumatic fever, a flare-up of disease activity frequently occurs, manifested by clinical abnormalities or by changes only in laboratory tests. The nature of this rebound phenomenon was recently analyzed in a retrospective survey of 265 patients with acute rheumatic fever (1, 2). The results indicated that most clinical rebounds were not due to any of the three possible causes to which they have been attributed in the past: *1*) polycyclic rheumatic activity, *2*) an insufficient duration of therapy or *3*) relative hypoadrenalism. Instead, the data strongly suggested that these rebounds represented the appearance of the inflammation that had been suppressed during the antirheumatic therapy. This hypothesis was based on the findings that the incidence of clinical rebounds increased with greater severity of cardiac involvement, that rebounds were more frequent in patients treated with steroids alone than in those treated with salicylates, that rebounds were more likely to occur after long courses of steroid therapy than after short courses, that they were rare after long courses of salicylates, and finally, that the incidence of post-steroid rebounds could be reduced by adding salicylates when the steroid reduction began, and by continuing salicylates for several weeks after steroids were stopped.

This new hypothesis has important implications regarding the natural history of rheumatic fever and has practical significance for its treatment. One of the implications has been used in the present study to provide a method of testing the validity of the theory. If the hypothesis is correct, it should be possible to "create" a rebound by giving anti-inflammatory therapy at a time when the convalescent rheumatic patient has no clinical abnormalities but is still in a subclinical inflammatory state. The use of profoundly suppressive therapy at this time might permit the remaining suppressed inflammation to reach an amount large enough so that clinical abnormalities occur when the treatment is discontinued. The occurrence of such an event had been noted previously (2) in one asymptomatic patient who was inadvertently given a short course of steroid therapy several weeks after his previous treatment had been discontinued and who had a clinical rebound when the steroid treatment was stopped.

The present work was undertaken as a prospective study of these phenomena in an effort to confirm or reject the proposed theory. The results, as noted below, confirm the new hypothesis.

CLINICAL MATERIAL AND METHODS

The patients ranged in age from 4 to 18 years and had all been admitted to Irvington House during the period beginning April 1959 for either acute or convalescent care of an attack of rheumatic fever which, in each instance, fulfilled the modified Jones diagnostic criteria (3). Because the initial course of anti-inflammatory therapy was usually begun elsewhere, the patients had been treated in different ways and for different lengths of time. Some had received only salicylates and some only steroids. Some had received "overlap" therapy in which salicylates were added to steroids and continued after the steroids were stopped, while others had received "combined" treatment in which steroids and salicylates were used together in a variety of ways other than the "overlap" technique.

After admission to Irvington House, anti-inflammatory treatment was begun, altered or concluded according to the individual clinical situation. In general, treatment was concluded within 12 weeks after onset of the acute rheumatic attack. In addition, the patients received oral prophylaxis daily, with sulfadiazine, 1.0 g, or with potassium penicillin G, 200,000 U, to prevent streptococcal infections. The absence of such infections during the present study was assured by periodic throat cultures and measurement of streptococcal antibodies.

After their initial treatment was stopped, some patients may have had a reappearance of rheumatic activity with laboratory or clinical abnormalities, and the

* Work performed in part during tenure of a Fellowship from the New York State Department of Health.

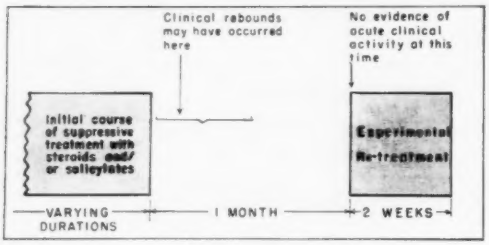
Clinical rebounds may have occurred here
No evidence of acute clinical activity at this time
Initial course of suppressive treatment with steroids and/or salicylates
Experimental Re-treatment
VARYING DURATIONS
1 MONTH
2 WEEKS

FIG. 1. PLAN OF EXPERIMENTAL THERAPY USED IN PRESENT STUDY.

latter sometimes received additional treatment before these derangements subsided. Patients became eligible for the present study after they had been without any anti-inflammatory therapy for 1 month. Although they may have had rebounds during that interval, they invariably had no *clinical* symptoms or signs of rheumatic activity when the experimental study began. The 1-month interval was chosen because of previous demonstrations (1) that post-therapeutic rebounds usually occurred within 2 weeks and were rare beyond 4 weeks after cessation of therapy. Therefore, when the experimental study was begun, all patients were beyond the "clinical rebound period" of their initial course of therapy. They were convalescent, asymptomatic and free of *clinical* evidence of acute rheumatic activity.

The plan of the experiment is shown in Figure 1. The experimental treatment was begun exactly 1 month after the previous anti-inflammatory treatment was stopped, and was continued for 2 weeks with one of the following regimens: *1*) prednisone, 60 mg per day for 1 week and 40 mg per day for the second week; *2*) aspirin, ¾ grain per lb per day for 1 week and ½ grain per lb per day for the second week; *3*) no therapy (control group).

For allocation into the experimental therapeutic groups, the patients were divided into three clinical categories. One group was considered to have "no valvular involvement" because they lacked significant cardiac murmurs, according to criteria which have been described elsewhere (1, 4, 5). The second group of patients had "valvular involvement" with no significant cardiac enlargement, i.e., no chamber more than 1+ enlarged. The third group had "valvular involvement" with significant cardiac enlargement. After classification into one of the three cardiac categories, each patient was allocated consecutively to one of the three experimental therapeutic groups. Because the number of patients was relatively small and because the consecutive order of allocation to the three therapeutic groups was followed rigorously within the clinical categories, the use of statistical random-number tables was not considered necessary. The plan for assignment of experimental therapy did not classify patients according to their previous treatment because the variations in its agent(s) and duration were too great. The most effective randomization seemed to be provided by considering the cardiac status and by maintaining the common property of being therapy-free for 1 month before onset of experimental treatment.

During the study the patients were observed for changes in clinical status and vital signs. Laboratory measurements were made of hematocrit, white blood cell count, sedimentation rate (Westergren and Wintrobe, uncorrected), serum C-reactive protein, and occult blood in

TABLE 1

Status of patients before experimental treatment

Cardiac status	Previous treatment	No. of patients	Types of post-therapeutic rebounds		
			None	Laboratory	Clinical
No valvular involvement	Steroids or "combined"	18	3	11	4
	Salicylate	12	10	1	1
	"Overlap"	2	1	1	0
	Subtotal	32	14	13	5
Valvular involvement without significant cardiomegaly	Steroids or "combined"	14	5	5	4
	Salicylate	14	6	5	3
	"Overlap"	7	2	4	1
	Subtotal	35	13	14	8
Valvular involvement with significant cardiomegaly	Steroids or "combined"	11	1	4	6
	Salicylate	5	1	2	2
	"Overlap"	5	1	4	0
	Subtotal	21	3	10	8
Entire group	Steroids or "combined"	43	9	20	14
	Salicylate	31	17	8	6
	"Overlap"	14	4	9	1
	Total	88	30	37	21

TABLE II

Relationship of clinical status and types of rebounds after 2 weeks of experimental suppressive therapy in patients with subsiding rheumatic fever *

Clinical status	Experimental therapy	No. of patients	Types of rebounds after experimental therapy: No rebound	Laboratory rebound	Clinical rebound
No valvular involvement	Prednisone	11	3	5	3
	Aspirin	10	10	0	0
	Control	11	10	1	0
	Subtotal	32	23	6	3
Valvular involvement without significant cardiomegaly	Prednisone	11	4	5	2
	Aspirin	12	11	1	0
	Control	12	10	2	0
	Subtotal	35	25	8	2
Valvular involvement with significant cardiomegaly	Prednisone	7	0	3	4
	Aspirin	7	4	2	1
	Control	7	7	0	0
	Subtotal	21	11	5	5
Entire group	Prednisone	29	7	13	9
	Aspirin	29	25	3	1
	Control	30	27	3	0
	Total	88	59	19	10

* Treatment given 1 month after cessation of previous therapy.

the stools (by guaiac test). These observations were made before, during, and after the experimental period of therapy. Three-position chest X-rays with barium were taken before and after the experimental therapy.

The criteria used for post-therapeutic clinical and laboratory rebounds have been defined elsewhere (1, 2) and will be reviewed here only briefly: A laboratory rebound consisted of an elevation either in sedimentation rate (over 20 mm per hour) or in C-reactive protein (trace or higher), without any clinical abnormalities. A clinical rebound was a laboratory rebound with associated clinical abnormalities. These could be manifested by fever, sleeping tachycardia, joint symptoms, or new features of carditis.

RESULTS

Status of patients before experimental therapy (Table I). Table I shows the situation of the present group before the beginning of experimental treatment. In the entire group, the initial treatment was followed by clinical rebounds in 6 of 31 patients who received salicylate, in 14 of 43 who received steroids or "combined" treatment, and in 1 of 14 treated with the "overlap" regimen. The incidence of clinical rebounds increased with increasing severity of cardiac damage. Laboratory rebounds were common, and bore somewhat similar relationships to the clinical state and the therapeutic agent. The results noted in this group are almost identical with those observed in other patients during earlier surveys (1, 2) of rebound phenomena and demonstrate that while no initial course of suppressive treatment was entirely able to avoid post-therapeutic clinical rebounds, the latter were more frequent after steroids and less frequent when salicylates were given alone or in such a way as to "overlap" the steroids.

Incidence of rebounds after experimental therapy (Table II). There were 88 patients in the study. Of the 29 who were experimentally treated with prednisone, 9 had clinical rebounds and 13 had laboratory rebounds after prednisone was discontinued. Of the 29 patients treated with aspirin, 1 had a post-therapeutic clinical rebound and 3 had laboratory rebounds. In the 30 control patients, there were no clinical rebounds and 3 laboratory rebounds.

Within the cardiac categories, the incidence of post-prednisone rebounds was the same in patients with no valvular involvement as in those who had valvular involvement without significant cardiomegaly (3 of 11 in the first group and 2 of 11 in the second). No clinical rebounds occurred in the

salicylate or control groups for either of these two clinical categories. In the group with significant cardiomegaly, the short course of salicylate was followed by a clinical rebound in 1 of 7 patients, while prednisone therapy provoked a clinical rebound in 4 of 7 patients. No clinical rebound occurred in the untreated group.

Within the experimentally treated prednisone group, therefore, the incidence of clinical rebounds was greater in patients with cardiomegaly than in those with no or slight heart damage. Aspirin therapy had effects significantly different from those in the control group *only* in patients with significant cardiac enlargement.

It is apparent that the short course of experimental therapy was able to induce clinical rebounds in patients who might otherwise have continued their course without complication if left untreated. The features of these clinical rebounds and the number of patients in whom they occurred were as follows: fever, 5; tachycardia, 1; fever and tachycardia, 1; fever, tachycardia and erythema marginatum, 1; arthralgia, 1; congestive heart failure, 1. All these manifestations subsided spontaneously without retreatment except for the congestive failure. The latter occurred in a patient with cardiomegaly who received prednisone; its clinical features disappeared with digitalis and diuretics.

Correlation between the previous post-therapeutic rebounds and those which followed the experimental course of therapy (*Table III*). Table III shows the relationship between the events in the 1-month interval which preceded experimental therapy and the events which followed it. In patients who had no rebound after their *previous* course of treatment, experimental therapy was not able to provoke a clinical rebound, although laboratory rebounds occurred in 6 of the 13 patients who received prednisone, in 1 of 8 who received salicylate and in 1 of the 9 controls. In patients whose *previous* therapy was followed by a laboratory rebound, the experimental course of salicylates produced essentially the same results as those seen in the control group: no clinical rebounds occurred and only 2 of the 26 patients had laboratory rebounds after the experimental period. By contrast, 6 of the 11 patients in this category who were treated with steroids had clinical rebounds and the other 5 had laboratory rebounds.

In the group of patients whose previous course of therapy was followed by a clinical rebound, there were no clinical rebounds in the controls. Subsequent clinical rebounds occurred in 1 of the 9 who were experimentally treated with salicylates, and in 3 of the 5 patients who received steroids.

These data show that the experimental retreatment with prednisone provoked either a laboratory rebound or a clinical rebound in all patients who had had evidence of rheumatic activity after their previous course of therapy. It pro-

TABLE III

Correlation between rebounds which followed the initial course of therapy and those which followed the experimental 2-week course of therapy 1 month later

Nature of rebound after initial course of therapy	Experimental therapy	No. of patients	Types of rebounds after experimental therapy		
			None	Laboratory	Clinical
None	Prednisone	13	7	6	0
	Aspirin	8	7	1	0
	None	9	8	1	0
	Total	30	22	8	0
Laboratory	Prednisone	11	0	5	6
	Aspirin	12	11	1	0
	None	14	13	1	0
	Total	37	24	7	6
Clinical	Prednisone	5	0	2	3
	Aspirin	9	7	1	1
	None	7	6	1	0
	Total	21	13	4	4
Total		88	59	19	10

TABLE IV

Relationship of previous therapy to experimental therapy and the rebounds after experimental therapy

Previous therapy	Experimental therapy	No. of patients	Types of postexperimental rebounds		
			None	Laboratory	Clinical
Steroids or "combined"	Prednisone	16	3	5	8
	Aspirin	14	12	1	1
	None	13	12	1	0
	Subtotal	43	27	7	9
Aspirin	Prednisone	9	3	6	0
	Aspirin	10	9	1	0
	None	12	10	2	0
	Subtotal	31	22	9	0
"Overlap"	Prednisone	4	1	2	1
	Aspirin	5	4	1	0
	None	5	5	0	0
	Subtotal	14	10	3	1
	Total	88	59	19	10

voked a clinical rebound in more than half of the patients whose first course of treatment was followed by only a laboratory rebound. These effects did not appear after the experimental treatment with salicylate. In this group, laboratory rebounds occurred in only 3 patients and clinical rebound in only 1. With the exception of the latter patient, the results of the experimental treatment with salicylate did not differ significantly from those of the control group. By contrast, none of the experimental therapeutic regimens was able to alter the course of the disease when rheumatic activity had subsided completely without rebound after the previous course of therapy.

Relationship of previous therapy to experimental therapy and postexperimental rebounds (Table IV). This table shows the occurrence of rebounds after experimental therapy in relation to the therapy which had been given before the experimental period. The three experimental regimens were divided about equally among the patients in each of the three "previous therapy" groups. Of the 43 patients who had been previously treated with steroid or "combined" therapy, 9 had clinical rebounds after the experimental treatment. The only salicylate-induced clinical rebound in this study occurred in 1 of these patients. Of the 31 patients treated with salicylate only, none developed clinical rebounds in response to experimental treatment. One of the 14 "overlap" patients had a clinical rebound after the subsequent course of prednisone.

Table I shows that clinical or laboratory rebounds may have occurred after the initial course of therapy in all three types of therapeutic regimens, less frequently in the salicylate group. Table IV shows that patients treated initially with salicylate failed to develop clinical rebounds after experimental therapy and that the greatest susceptibility to the experimental rebounds was in patients treated initially with steroids or "nonoverlapping" combined therapy.

Effect of experimental treatment upon the erythrocyte sedimentation rate (Figure 2). With minor numerical differences, the Westergren and uncorrected Wintrobe sedimentation rates showed identical variations. For convenience of illustration the Wintrobe rates have been used in Figure 2.

Using a value of 20 mm per hour as the upper limit of normal, the sedimentation rates in the "no valvular involvement" group were abnormally elevated before experimental therapy in 3 of 11 controls, in 4 of 10 patients who received aspirin, and in 3 of 11 treated with prednisone. In the patients with noncardiomegalic "valvular involvement," the pretreatment sedimentation rate was elevated as follows: controls, 5 of 12; aspirin, 6 of 12; prednisone, 4 of 11. In the group with significant cardiomegaly, an abnormal pretreatment sedimentation rate was found as follows:

controls, 5 of 7; aspirin, 4 of 7; prednisone, 4 of 7.

The pretherapeutic values of the sedimentation rate and the response to experimental treatment are shown in Figure 2. In all three clinical categories of the control group, the erythrocyte sedimentation rate (ESR) remained the same or showed only slight changes. During the experimental salicylate treatment, the ESR rose in 9 patients and fell when treatment was discontinued; in the other patients it remained the same or declined slightly. [This paradoxical rise in sedimentation rate with aspirin has been noted in the past (6). It appeared mainly in patients who showed some gastric intolerance of the drug. Its mechanism is obscure; it may be related to gastritis or to occult bleeding.] By contrast, the ESR fell during treatment in all prednisone patients, regardless of its initial value. In almost every instance, the ESR after treatment was higher than its pre-prednisone value. These data suggest that steroid therapy suppresses inflammation profoundly (as measured by sedimentation rate) and is followed by recrudescence of the inflammation to levels greater than those which existed previously.

The heavy black lines in Figure 2 show the course of the patients who developed clinical rebounds after the experimental therapy was stopped. In 6 of these 10 patients the sedimentation rate was above 20 before experimental treatment and in 8 it was above 20 at the time of the clinical rebound. The 2 patients without a high sedimentation rate at that time had abnormal values for serum C-reactive protein. The variations in reaction of these parameters presumably reflect the

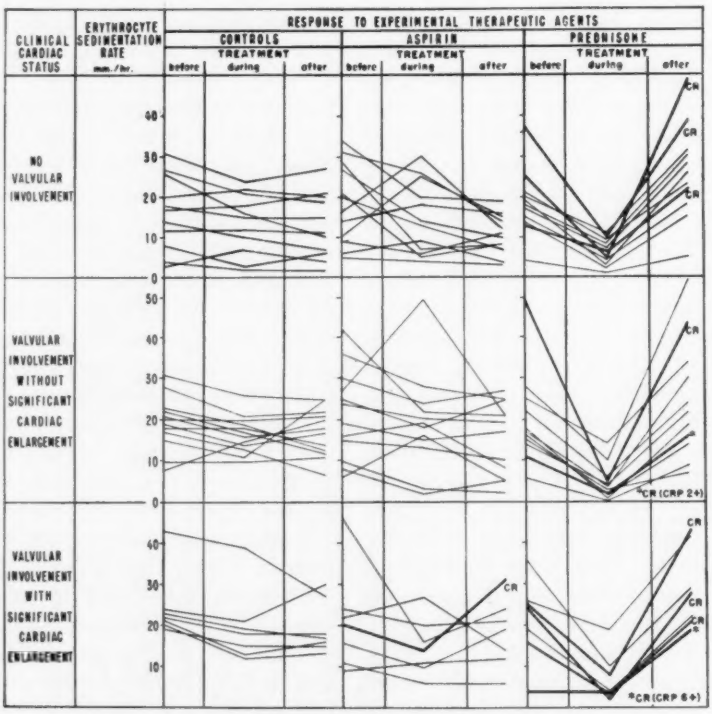

FIG. 2. RESPONSE OF SEDIMENTATION RATE TO EXPERIMENTAL THERAPY AND APPEARANCE OF POST-THERAPEUTIC CLINICAL REBOUNDS IN PATIENTS CONVALESCENT FROM RHEUMATIC FEVER. Sedimentation rate is the uncorrected Wintrobe value. CR = clinical rebound. Heavy black lines used for patients with clinical rebounds. CRP = serum C-reactive protein value, shown for patients whose sedimentation rate was below 20 at time of clinical rebound.

different capacities with which they measure the existence of inflammation in different patients.

Effect of experimental treatment upon the serum C-reactive protein (Table V). The values found here parallel those observed with the sedimentation rate. Of the 29 patients receiving prednisone, 2 had an abnormal value before the experimental therapy, none had an abnormal value during therapy and 6 had abnormal tests afterward. Five patients on salicylate treatment had a pretherapy elevation in C-reactive protein which became normal thereafter. In 7 patients, abnormal values for C-reactive protein appeared during therapy but were negative previously and afterward. The effect of salicylate in elevating this acute phase reactant is consistent with its effects, noted simultaneously, upon sedimentation rate and with those noted in earlier experiments with serum transaminase (7). In the control group, 3 patients had abnormal values before treatment, 1 during treatment and none thereafter.

Changes in hematocrit during and after experimental treatment (Table VI). The hematocrit values during and after treatment were compared in all patients with those which were present before the experimental treatment began. The changes are listed in Table VI. In the control group there was no essential difference during or after treatment. The mean hematocrit of the salicylate group fell during treatment while that of the prenisone group rose. Both mean changes, compared with those of the control group were statistically significant. Within 1 to 2 weeks after stopping the experimental treatment, the hematocrit returned to its previous levels with no significant difference between the treatment groups and the controls.

TABLE V

Behavior of C-reactive protein in relation to experimental therapy

Experimental therapy	No. of patients	No. of patients with abnormal C-reactive protein		
		Before therapy	During therapy	After therapy
Prednisone	29	2	0	6
Aspirin	29	5*	7†	1
Control	30	3	1	0

* The 5 patients with pretherapy elevations in C-reactive protein had no abnormalities thereafter.

† The 7 patients whose abnormal C-reactive protein appeared during therapy did not have it previously.

TABLE VI

Changes in hematocrit during and after experimental treatment, compared with pretreatment value

Treatment	No. of patients	Mean changes in hematocrit	
		During treatment	After treatment
Prednisone	29	+3.3* ± 3.2	−0.2† ± 3.3
Aspirin	29	−1.4* ± 3.1	+0.1† ± 2.1
Controls	30	0 ± 2.1	−0.1 ± 1.5

* The difference between these means and that of the controls is more than twice the standard error of the sampling.

† The difference between these means and that of the controls is *not* more than twice the standard error of the sampling.

Changes in white blood cell count during experimental treatment; other changes. As was expected (8), all patients receiving the experimental course of prednisone had an elevation in white blood cell count during the 2-week treatment period; the white counts returned to their previous levels shortly after treatment was stopped. Salicylate had no effect upon the white count.

The stool guaiac test became positive in 2 patients who received salicylate therapy and became negative after treatment was stopped. There were no abnormalities in the stool guaiac tests of patients who received prednisone or in the control group. There were no significant changes in the cardiac size of any patients, during or after therapy, as measured by three-position chest X-rays.

DISCUSSION

These data provide the first evidence that rebounds of clinical activity in rheumatic fever can be deliberately produced by the use of an apparently benign therapeutic procedure. A 2-week course of anti-inflammatory therapy, given to asymptomatic rheumatic patients in a convalescent state after an acute attack, was followed by a recrudescence of clinical activity in 9 of 29 patients who received prednisone and in 1 of 29 who received aspirin. The untreated control group remained asymptomatic. Clinical rebounds after the experimental therapy occurred only in patients who had shown some clinical or laboratory evidence of rheumatic activity following their original course of suppressive treatment and only in pa-

tients whose original treatment included steroids.

The results have several sets of implications:

A. They help confirm, and can be explained only by, the hypothesis that a rheumatic post-therapeutic rebound represents the reappearance of the inflammation which was previously suppressed. The alternative explanations for the events noted in the present study and the evidence against them are as follows: *1*) Recurrent episodes of rheumatic fever did not cause the flare-ups because there had been no associated streptococcal infections. *2*) Polycyclic rheumatic activity was unlikely, since it did not appear in the control group and because the flare-ups noted here were so heavily localized in the prednisone group. *3*) A nonspecific reaction, perhaps hypoadrenalistic, to steroid cessation is an unlikely explanation for these reasons: *a*) a clinical rebound occurred in a salicylate-treated patient; *b*) the rebounds were more frequent in patients with more severe heart disease; *c*) in two patients the rebounds had clinical features characteristic of acute rheumatic fever —erythema marginatum and congestive heart failure—and were not merely *nonspecific* inflammatory responses; and *d*) the experimental post-steroid symptoms did not occur randomly but appeared only in patients who had had some form of rebound previously after their initial course of therapy. Patients who had had no evidence of rheumatic activity after their initial therapy were clinically unresponsive to the experimental steroid treatment.

B. The explanation which best fits these and previous (1, 2) data is that an attack of rheumatic fever is associated with a stimulus or substance which produces inflammation and which is then consumed or dispersed in the inflammatory process. The usual course is for the inflammation to create clinical manifestations at first, to become subclinical later, and finally to cease. In most attacks of rheumatic fever, there seems to be a finite amount of this hypothetical substance; it creates a finite amount of inflammatory reaction and is dispersed by it in a finite length of time. The amount of this substance, and hence of the inflammation, will vary with individual rheumatic attacks and with individual patients, and will determine the severity of the attack, the residual cardiac damage, and the duration of measurable abnormalities (9) in laboratory tests.

Therapy with steroids or salicylates or both will generally suppress the inflammation and will reduce the severity of its clinical and laboratory manifestations. Although the expression of inflammation may be suppressed, the underlying disease remains active and the production of the hypothetical substance continues. The salicylates or subtherapeutic doses of steroids will suppress inflammation only partially, so that it is manifested by laboratory abnormalities without associated clinical features. During the period of partial suppression, some of the hypothetical substance can be dispersed as the subclinical inflammation proceeds. When the suppression of inflammation is total, as with large doses of steroids, little or no dispersion occurs and the substance accumulates. When the total or partial suppression ceases, the clinical or laboratory magnitude of the inflammation which then appears will depend upon how much of the substance remains to be dispersed.

This hypothesis explains the types of rebounds seen in earlier surveys (1, 2) of initial treatment of rheumatic fever. It also explains the present findings that profound suppression of rheumatic inflammation, at a time when it has reached subclinical levels but has not yet ceased, may be followed by clinical manifestations later when the inflammatory reaction resumes. These concepts appear to apply equally well to the rebound events noted in treatment of acute thyroiditis (10) and invite analogy regarding autoimmune phenomena in rheumatic fever.

C. These concepts do not apply to a small percentage of severely ill patients with rheumatic fever who show "chronic" or unusually prolonged clinical rheumatic activity (11) with spontaneous remissions and recrudescences unrelated to preceding therapy. There were no such patients in the present study.

D. Finally, the data indicate that anti-inflammatory drugs, particularly steroids, should *not* be given to patients with rheumatic fever who do *not* show acute clinical abnormalities, since the therapy itself may provoke a recrudescence of clinical activity and iatrogenic "chronicity" of the disease. This is particularly pertinent in situations where resumption of therapy is contemplated because its initial cessation was followed by abnormalities only in acute phase reactants. Previous work has

shown that these "laboratory rebounds" subside spontaneously (1, 2) and the present data show that their treatment may provoke clinical rebounds.

SUMMARY

A 2-week course of treatment with prednisone or aspirin was given experimentally to clinically asymptomatic patients who were convalescing from acute rheumatic fever. The experimental treatment was begun exactly 1 month after the initial course of suppressive treatment was ended. When the experimental therapy was stopped, a recrudescence of clinical rheumatic activity occurred in 9 of 29 patients treated with prednisone, in 1 of 29 treated with aspirin and in none of 30 controls. The experimental clinical rebounds, which were more frequent in patients with severe cardiac damage, occurred only in patients who had shown clinical or laboratory evidence of rheumatic activity after their previous course of treatment and only in those whose previous treatment included steroids.

The experimental treatment with prednisone caused a fall in the sedimentation rate of all patients (regardless of its initial value) and its discontinuation was followed by a rise, often to greater than pretreatment levels. It elevated the white blood cell count in all patients and caused a statistically significant rise in mean hematocrit values. The experimental treatment with aspirin caused paradoxical rises in sedimentation rate and C-reactive protein in about one-fourth of the patients; it produced positive stool guaiac tests in 2 of 29 patients and a statistically significant fall in mean hematocrit of the group.

The data are consistent with, and help confirm, the hypothesis that the post-therapeutic rebound phenomenon in rheumatic fever occurs because suppressive therapy, in preventing or inhibiting the inflammatory process, also prevents or inhibits the dispersion of the stimulus that causes inflammation. The subsequent removal of the suppressive agent is followed by the resumption of inflammation in magnitude proportional to the remaining causative stimulus.

The results indicate that anti-inflammatory treatment should not be begun or resumed for patients with rheumatic fever whose acute abnormalities are only in laboratory tests.

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A LIPID–PROTEIN FRACTION IN RHEUMATOID PLASMA PRECIPITABLE WITH CHONDROITIN SULFATE AFTER EUGLOBULIN REMOVAL *

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Acid mucopolysaccharides of human plasma have been observed (1) to vary as do acute phase reactants, i.e., to increase nonspecifically in any inflammatory state. The euglobulin fraction itself with which the acid mucopolysaccharides were associated increased also in such circumstances. On later comparing levels of acid mucopolysaccharides in samples of serum and plasma drawn simultaneously, it was noted that the acid mucopolysaccharides measurable in the euglobulin fraction were derived at least in part from the formed elements of blood during blood clotting (2). It seemed possible, therefore, that the amount of plasma protein precipitable as euglobulins by dilution at pH 5.8 might increase in inflammation as a result of the release of acid mucopolysaccharides into the blood, causing alteration of isoelectric point of plasma proteins already present. Precedent for such a possibility has been well established by the earlier observations of other investigators on the interactions of fibrinogen and β-lipoproteins and polyanions (3, 4). The possibility as it relates to rheumatoid plasma was mentioned in effect also by Badin, Schubert and Vouras (5).

To explore this possibility, studies were carried out on the interaction of chondroitin sulfate with the proteins remaining in plasma after isoelectric precipitation of spontaneously occurring euglobulins by dilution with mineral acid to pH 5.8. The additional protein available for precipitation under identical conditions of dilution and pH on addition of chondroitin sulfate (the fraction being called S_1) was strikingly increased by prior dialysis of the plasma against balanced bufferred salt solution. This increase was prevented if a heat-resistant, organic, dialyzable factor present in a pressure dialysate of plasma was restored to the dialyzed plasma prior to use. The S_1 fraction had a high cholesterol content. The amount available for precipitation from nondialyzed plasma of rheumatoid patients considerably exceeded that of patients without rheumatoid disease, with or without other inflammatory processes.

METHODS

In dialysis experiments, oxalated plasma was dialyzed at 5° C for 21 to 27 hours in Visking 18/32-inch cellulose casing against 20 to 50 vol of balanced buffered salt solution.[1] Control plasma was held at 5° C for equal time and before use was made to volume (with balanced buffered salt solution) exactly equal to that of the dialyzed plasma (which increased in volume slightly during dialysis).

In surveying patients, oxalated blood for plasma was obtained from three groups of individuals, as previously defined in detail (1). Briefly, group I showed no evidence of inflammatory disease and had negative C-reactive protein tests; group II had significant inflammatory disease unrelated to rheumatoid or other primary connective tissue disease; and group III had clinically unequivocal active rheumatoid arthritis, in no instance being treated with hormones.

Euglobulins were precipitated from all plasma samples by the mineral acid dilution method used previously (1), and described briefly in Figure 1. Chondroitin sulfate (General Biochemicals, Inc.) was added, 2 mg per ml of physiological saline in volume one-half that of the original plasma, to the supernatant diluted plasma, to obtain the S_1 fraction. The entire method of recovering fractions is summarized schematically in Figure 1. The protein content of euglobulin and S_1 fractions was determined by biuret reaction (6), using human serum albumin as a standard and expressing content as milligram equivalence per 100 ml of original plasma. The cholesterol content of the fractions was determined after extraction with acid isopropyl alcohol and heptane (7), by the method of Zlatkis, Zak and Boyle (8). Results were expressed as milligrams per 100 ml of original plasma. A standard curve of 10, 25, 50, 100 μg of puri-

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[1] Ten ml of stock solution (7.5 per cent NaCl, 0.75 per cent KCl, 0.1 per cent Na_2HPO_4, 0.12 per cent KH_2PO_4, 0.05 per cent K_2HPO_4) and 7 ml of 1 per cent Na_2HPO_4 per 100 ml, aqueous (total phosphate concentration, 6.7×10^{-3} M).

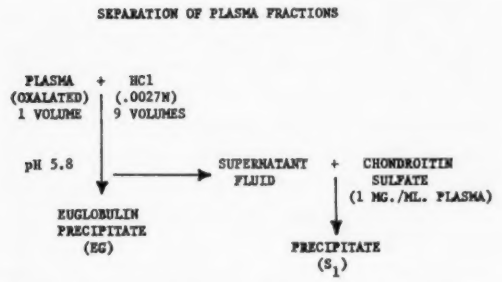

FIG. 1. SCHEMATIC SUMMARY OF PLASMA FRACTIONATION METHODS USED. Carried out at 5° C.

field cholesterol was run in duplicate with every set of determinations. All plasma fractions were derived and run in duplicate both for protein and cholesterol content, with close checks easily obtained.

RESULTS

Table I illustrates the striking increase in protein available for precipitation in the S_1 fraction after dialysis of the original plasma. This was a consistent finding with all plasmas. The difference was gross, as shown in Figure 2 which compares the appearances of (*a*) S_1 fraction from nondialyzed plasma, (*b*) the same plasma after dialysis,

TABLE I

Effect of dialysis of plasma on the protein (biuret) and cholesterol content of subsequent euglobulin and S_1 fractions

Noninflammatory plasma	Biuret	Cholesterol
	mg/100 ml plasma	
Nondialyzed:		
Euglobulins	442	10.0
S_1 fraction	259	2.0
Dialyzed:*		
Euglobulins	423	22.0
S_1 fraction	742	45.0+

* Dialyzed against 20 vol balanced buffered salt solution, pH 7.4, for 21.5 hours at 5° C.

and (*c*) dialyzed plasma to which a small amount of undiluted plasma dialysate (obtained by centrifuging plasma in Visking cellulose casing for 8 to 9 hours at 5° C at 1,000 G) was added before fractionation of the plasma. The factor (or factors) which restored the dialyzed plasma to its nondialyzed state in regard to S_1 precipitability was dialyzable, resisted heating to 100° C for 15 minutes, but was destroyed by ashing. Addition of certain known dialyzable plasma components and other substances to the buffered balanced

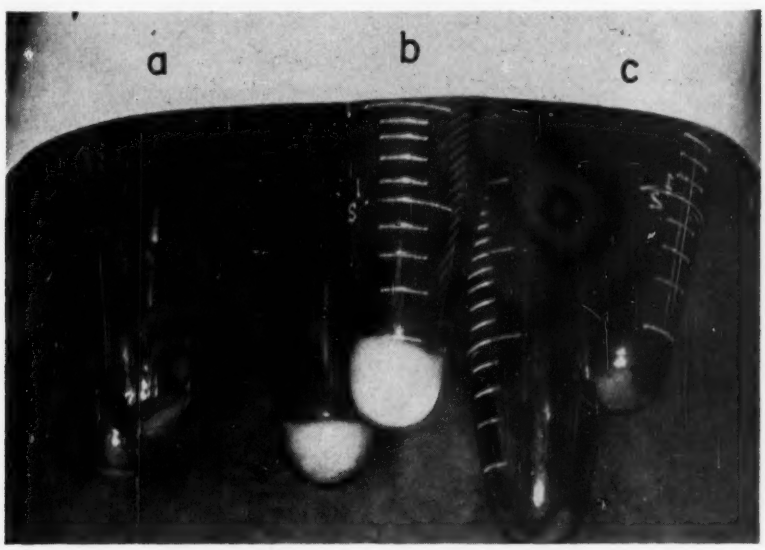

FIG. 2. GROSS EFFECT OF PLASMA DIALYSIS ON SUBSEQUENT S_1 FRACTION RECOVERED. S_1 fractions (in duplicate) centrifuged at 5° C and photographed after decantation of the supernatant fluid. (a) S_1 from original nondialyzed plasma. (b) S_1 from same plasma after dialysis. (c) S_1 from same plasma after dialysis and then addition of a plasma pressure dialysate before fractionation.

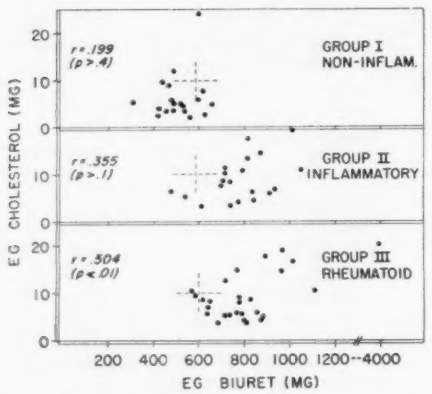

FIG. 3. CORRELATION DATA ON EUGLOBULIN FRACTIONS, CHOLESTEROL AND PROTEIN (BY BIURET) CONTENT (IN MG PER 100 ML ORIGINAL PLASMA). r = Correlation coefficient. The dotted crossed lines are arbitrarily and identically placed in the three boxes as visual reference aids.

salt solution before dialysis of plasma failed to prevent the subsequent increase in S_1 fraction. These included $(NH_4)_2SO_4$ (8.3×10^{-4}M), $MgCl_2$ (2×10^{-4}M), glucose (7×10^{-3}M), urea (3.3×10^{-3}M), uric acid (2.4×10^{-5}M), glutamic acid (1.4×10^{-4}M), and sodium salicylate (2.5×10^{-4}M). Restoration of Ca^{++} to the system was without effect, and serum could be substituted for plasma without apparent alteration of results. Prevention of loss of oxalate during dialysis (by use of equal concentration outside the casing) did not prevent the subsequent increase in S_1 fraction. On the chance that the dialysis effect simply reflected an alteration in labile lipoproteins, as noted on dialysis of plasma by Ray, Davisson and Crespi (9), the effect of Cu^{++} (1.5×10^{-5}M, 4×10^{-5}M), of serine (4.8×10^{-4}M) and of versene (0.1 per cent disodium EDTA) was studied exhaustively in the system, with failure to demonstrate any change such as Ray and co-workers described as a cause of the increased S_1 after dialysis of plasma. The amount of S_1 obtained appeared, indeed, maximal when versene was added (0.1 per cent) to the balanced buffered salt solution used in dialysis. This was precisely the opposite effect from that expected if the S_1 changes had been due to influences on lipoproteins of the type described by Ray and associates.

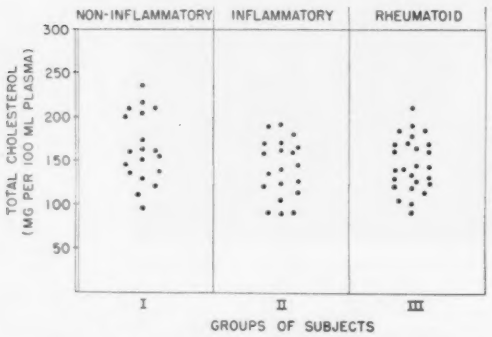

FIG. 4. TOTAL PLASMA CHOLESTEROL LEVELS.

In surveying plasma of patients for evidence of possible biological significance in the above-described observations *in vitro*, the euglobulin fractions were found to be increased in the inflammatory groups II and III, as expected from previous studies (1). The euglobulin data are summarized in Figure 3, which plots protein content of the fraction against its cholesterol content. Only the rheumatoid group III showed significant correlation between protein and cholesterol content of the euglobulin fraction. Neither the cholesterol content nor the percentage of cholesterol in the euglobulin fraction correlated with the total plasma cholesterol in any of the three groups. Figure 4 shows the similarity in range of total plasma cholesterol in the three groups of patients studied.

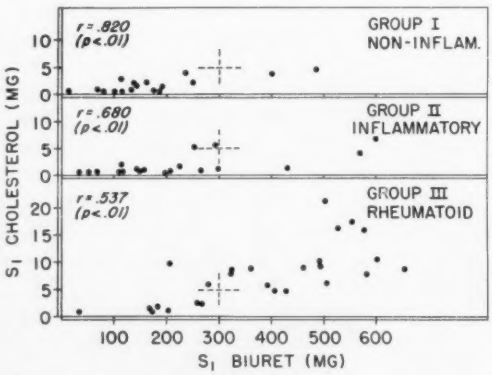

FIG. 5. CORRELATION DATA ON S_1 FRACTIONS, CHOLESTEROL AND PROTEIN (BY BIURET) CONTENT (IN MG PER 100 ML ORIGINAL PLASMA). Dotted crossed lines and r as in Figure 3.

Figures 5 and 6 summarize the S_1 data. Here there was high correlation between biuret and cholesterol content of the fraction in all three

groups (Figure 5). The amount of lipid-protein fraction available for precipitation as S_1 was considerably greater in rheumatoid group III than in either of the nonrheumatoid groups. In the nonrheumatoid groups I and II, those patients with the highest percentage of cholesterol in S_1 fraction tended also to be those patients with highest total plasma cholesterol (Figure 6). This correlation did not exist in the rheumatoid group III.

The S_1 fractions from the three groups of patients showed no qualitative differences between the groups, either by standard Ouchterlony plate techniques (rabbit antiserum to S_1 fractions versus Cohn fractions of human plasma) or by electrophoretic techniques. However, the quantitative similarity between the S_1 fraction from nondialyzed rheumatoid plasma and that from dialyzed normal plasma was again emphasized on paper electrophoresis (Figure 7). Precipitation bands were obtained with S_1 antiserum against both Cohn fractions I and III by Ouchterlony techniques, indicating that the S_1 fraction included fibrinogen and β-lipoprotein among its components.

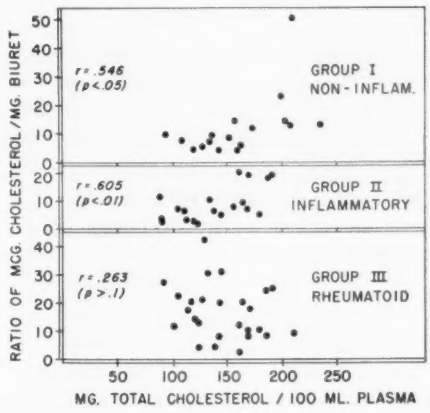

FIG. 6. CORRELATION BETWEEN PERCENTAGE CHOLESTEROL CONTENT OF S_1 FRACTIONS AND TOTAL PLASMA CHOLESTEROLS. r as in Figure 3.

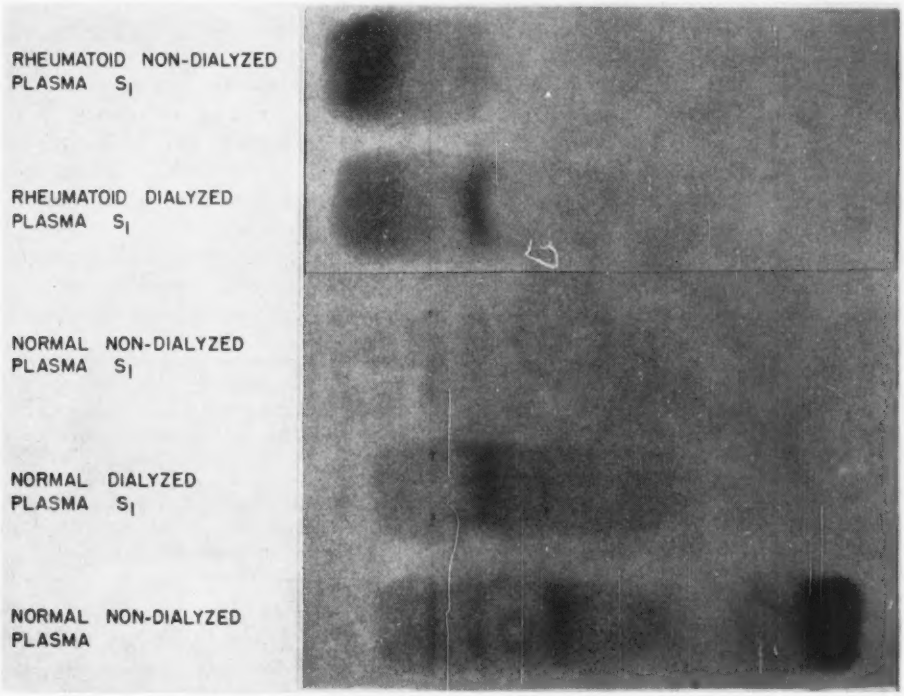

FIG. 7. PAPER ELECTROPHORESIS OF S_1 FRACTIONS, AS INDICATED. All S_1 fractions were concentrated twofold (taken up to one-half original plasma volume) for use here. The whole plasma was used at original concentration. Run at 10 ma for 7 hours on Whatman no. 3 MM paper in 0.12 M, pH 8.6, veronal buffer.

DISCUSSION

Previous studies (10) have shown that predominant spontaneous occurrence of acid mucopolysaccharides of human plasma was in Cohn fractions III and IV-1.[2] In the present studies, the lipid-protein fraction precipitable from any human plasma with chondroitin sulfate after removal of spontaneously occurring euglobulins but under identical conditions of dilution and pH (S_1 fraction) was found to be rather uniform and high in its cholesterol content. The spontaneously occurring euglobulin fraction of rheumatoid plasma, in contrast to that from nonrheumatoid patients with or without other inflammatory disease, showed uniformity and elevation of cholesterol content comparable with that of the S_1 fraction produced artificially by appropriate addition of chondroitin sulfate. The possibility can be considered therefore that the increased euglobulins in rheumatoid plasma may represent in significant part plasma protein, the isoelectric point of which has been shifted by association with a polyanion such as an acid mucopolysaccharide so that it precipitates under the *in vitro* conditions of dilution and pH which define precipitability of euglobulins.

A further difference between rheumatoid and nonrheumatoid plasma lay in the presence of significantly increased amounts of demonstrable S_1 fraction in rheumatoid plasma. Any plasma could be made to resemble rheumatoid plasma in this regard by first dialyzing out of it a heat-resistant, organic material which, in turn, could be re-added to dialyzed plasma before use to completely revert the plasma to its original nondialyzed state as far as subsequent S_1 precipitability was concerned.[3] Because of the resemblance of dialyzed plasma to rheumatoid plasma in this regard, an intensive effort is in progress to identify the dialyzable factor or factors involved. This effort has been furthered by finding that a similar if not identical factor can be demonstrated in both human urine and spinal fluid. Other quantitative studies in progress suggest that these findings may be of biological significance in rheumatoid disease. The presence of a dialyzable substance in biological materials capable of influencing the combination of acid mucopolysaccharides with proteins may offer a lead into the system of checks and balances which must certainly regulate this area of connective tissue physiology as similar systems regulate other biological processes such as blood coagulability.

SUMMARY

1. The protein remaining in human plasma after isoelectric precipitation of euglobulins was studied for precipitability with chondroitin sulfate under the same conditions of dilution and pH which precipitated euglobulins. The protein so precipitated was called the S_1 fraction. Cholesterol content of the lipid-protein S_1 fraction showed a positive correlation with protein content.

2. This S_1 fraction was significantly increased in the plasma of rheumatoid patients, in contrast to nonrheumatoids with or without other inflammatory disease.

3. Any plasma could be made to resemble rheumatoid plasma in this regard by first dialyzing it against balanced buffered salt solution.

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[2] The validity of this observation would appear to be unchallenged by the later observation of Epstein and Block (11) who noted that only 0.5 per cent of total plasma hexosamine was found in lipoprotein fractions of human plasma. By the data from this laboratory (1) and by those of Bollet (12) on total acid mucopolysaccharides, and recognizing rough equivalence by weight of hexuronic acid and hexosamine content in acid mucopolysaccharides, less than 0.4 per cent of the total plasma hexosamine as measured by Epstein and Block and by Bollet could be expected to be found in total acid mucopolysaccharides of human plasma, regardless of where they occurred fractionally.

[3] It is of interest that the euglobulin fraction of dialyzed plasma regularly showed an increase in acid mucopolysaccharide content when measured by methods detailed elsewhere (1) as compared with the euglobulin fraction of the same plasma nondialyzed. This occurred regardless of whether the over-all protein content of the fraction increased, decreased, or remained unchanged after dialysis. Vertically paired data (below) show the acid mucopolysaccharide content (expressed as micrograms of hexuronic acid per 100 mg euglobulin protein) of nondialyzed (ND) and dialyzed (D) plasma in several experiments:

ND	75	55	76	89	50	58
D	161	156	139	159	120	95

This was assumed to reflect again the change in isoelectric point of proteins influenced by attached small groups which could be dialyzed off the protein. The euglobulin fraction after dialysis appeared to consist of proportionally more acid-mucopolysaccharide-associated protein than before dialysis.

4. Addition of plasma dialysate to dialyzed plasma prior to its use reversed the dialytic effect. The factor in the dialysate which accomplished the reversal was resistant to heating to 100° C for 15 minutes but not to ashing.

5. Further studies are intensively in progress to identify the dialyzable material which was shown to affect the combination of acid mucopolysaccharides with plasma protein.

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THE URICOSURIA AND OROTIC ACIDURIA INDUCED BY 6-AZAURIDINE *

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The pyrimidine analog 6-azauridine (6-AZUR) interrupts the major pathway for the *de novo* biosynthesis of pyrimidine nucleotides after its biological conversion to 6-azauridine-5'-phosphate (6-AZUMP). 6-AZUMP is a competitive inhibitor of the enzyme which decarboxylates orotidylic acid (see Figure 1) (1, 2). 6-AZUR inhibits bacterial reproduction as well as tumor growth in animals (3–6). The latter effect has recently led to its use in the experimental treatment of human leukemia and solid tumors (7, 8).

Therapy with 6-AZUR in man, conducted at the National Cancer Institute, was occasionally complicated by a pronounced crystalluria. The crystals were separated into two components by ion-exchange chromatography and were identified by their ultraviolet absorption spectra as uric and orotic acid. The appearance of orotidine in tissues and orotic acid and orotidine in the urine of mice receiving 6-AZUR has been previously noted (1, 3) and may be explained by the metabolic block produced by the drug. These compounds have also been noted in the urine of humans receiving 6-AZUR or 6-azauracil by several investigators (9–12). More detailed study of the urinary excretion pattern of orotic acid and orotidine in response to 6-AZUR administration in the human forms one part of this investigation.

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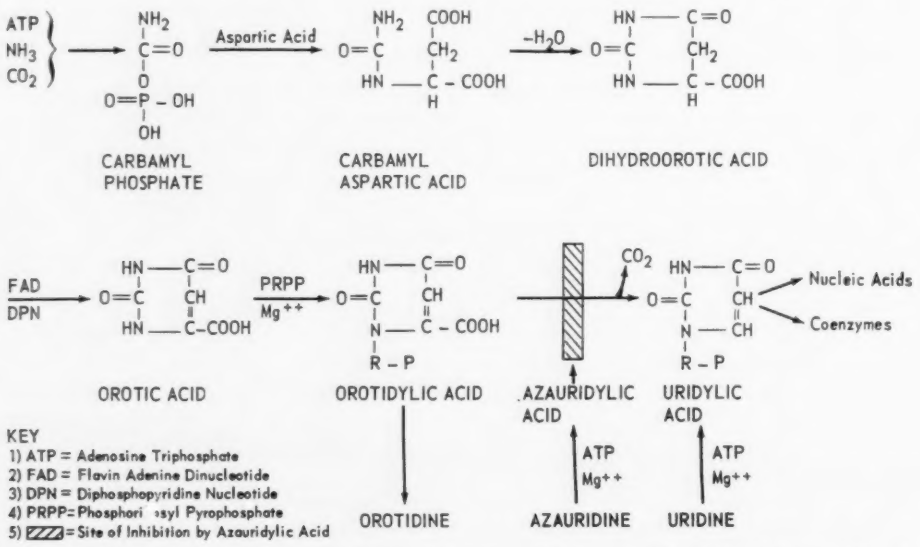

FIG. 1. *De novo* SYNTHESIS OF PYRIMIDINE NUCLEOTIDES. The main pathway leads from carbamylphosphate to orotidylic acid and then by decarboxylation to uridylic acid. A less important pathway for most mammalian tissues involves the conversion of uridine to uridylic acid. Azauridylic acid is formed from azauridine by a kinase reaction and inhibits the decarboxylation step.

TABLE I

The changes in serum and urinary urate of representative patients receiving Azauridine. Similar changes have occurred in all patients studied

Patient Diagnosis *	Urine Urate		Serum Urate		Duration of Therapy	Dose of 6-AZUR
	Average Before Therapy	First Day on Therapy	Average Before Therapy	Last Day on Therapy		
	Mg/24 hrs.	*Mg/24 hrs.*	*Mg %*	*Mg %*	*Days*	*Mg/Kg/day*
CML	798	2,953	9.5	2.4	20	120
CLL	552	949	4.2	2.6	17	120
CML	750	2,070	10.5	3.1	3	120
CML	1,170	2,145	5.9	2.6	5	60
P.V.	487	1,278	8.6	3.4	4	120
CLL	292	835	6.8	4.2	2	120

* CML – Chronic Myelogenous Leukemia
CLL – Chronic Lymphocytic Leukemia
P.V. – Polycythemia Vera.

The presence of urate crystalluria led to the demonstration of a decline in the serum urate levels and an increased urinary excretion of uric acid during therapy with 6-AZUR. This finding was totally unanticipated, and an attempt was made to determine whether the uricosuria induced by 6-AZUR resulted from a primary renal effect or was related to its metabolic effect and the accompanying orotic aciduria.

METHODS

Patients with nonterminal malignant disease who were to receive intravenous 6-AZUR therapy were placed on diets essentially free of purines and pyrimidines including orotic acid [1] for at least 5 days prior to and throughout their study period. Patients received 6-AZUR in four 2-hour infusion periods daily for 3 to 20 days and were maintained on a constant intravenous infusion of 5 per cent dextrose and water during the remainder of the day. For 12 to 24 hours prior to 6-AZUR or orotic acid administration patients received 1 to 1.5 L of 5 per cent dextrose in water as a control period to correct for any possible uricosuric effect of the large volumes of fluid used for the administration of 6-AZUR. All urine was refrigerated during collection with toluene as a preservative and aliquots were frozen for the subsequent determination of the daily excretion of orotic acid, orotidine and 6-AZUR. Creatinine and uric acid were determined on fresh urine specimens after warming to assure that any crystals were dissolved.

The effect of infusion of 6-AZUR on the renal clearance of inulin and urate was also determined in three patients in order to better define the mechanism of action of the drug. In addition, urate excretion during the infusion of orotic acid and orotidine was studied.

Preparation of infusions. 6-AZUR obtained from the Cancer Chemotherapy National Service Center was prepared in isotonic sodium bicarbonate at a concentration of 1 g per 10 ml and sterilized by filtration through Millipore filters (0.45 μ). Appropriate quantities of this material were than added to 150 ml of 5 per cent dextrose and water for intravenous infusion during a 2-hour period.

Orotic acid (California Corporation for Biochemical Research) was dissolved in 5 per cent dextrose and water at the concentration of 2 mg per ml by adjusting to pH 7 with sodium hydroxide. This concentration was found to be stable for several months at room temperature after sterilization by filtration (*vide supra*). More concentrated solutions tended to crystallize at room temperature. This material was infused without further dilution.

Orotidine (obtained through the courtesy of R. E. Handschumacher) was administered as the sodium salt in a concentration of 1 mg per ml of 5 per cent dextrose and water.

Analytical methods. The uric acid concentration of serum and urine was determined by the spectrophotometric method of Kalckar (14) as modified by Liddle, Seegmiller and Laster (15). Addition of orotic acid or 6-AZUR to urine did not inferfere with urate determinations by this method. Serum and urine creatinine was determined by the method of Bonsnes and Taussky (16). Inulin was determined as described by Walser, Davidson and Orloff (17).

The pyrimidine compounds were determined by a spectrophotometric method after separation by ion-exchange chromatography.[2] One-ml aliquots of urine, di-

[1] Milk- and milk product-free (13).

[2] Based on method of R. E. Handschumacher.

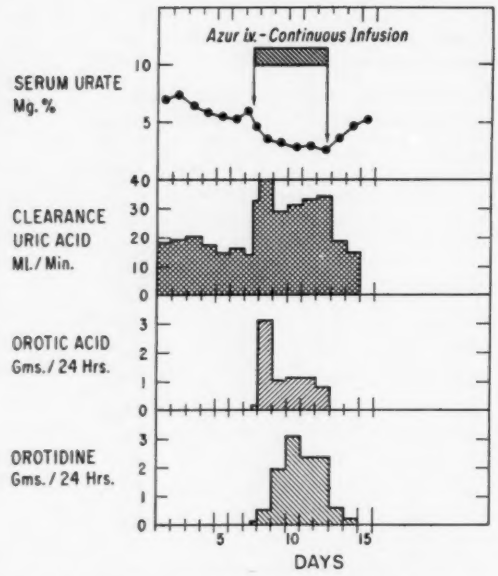

FIG. 2. INCREASE IN URINARY CLEARANCE OF URIC ACID DURING CONTINUOUS 6-AZAURIDINE INFUSION. The excretion pattern of orotic acid and orotidine shows the rapid peak in orotic acid excretion and the presence of orotidine in the urine for 48 hours following cessation of 6-azauridine.

luted 1 : 10 with 0.05 M ammonium formate, were passed through 5 ml of Dowex 1-X10 formate 200-400 mesh in 1 × 30 cm columns. The resin was washed with boiled distilled water for 6 to 8 hours both before and after application of the urine to reduce the background optical density. Ten-ml fractions of eluate were collected during successive elution with 30 ml of 0.05 N formic acid, 30 ml of 0.1 N formic acid, 10 ml of water and 150 ml of 0.3 N ammonium formate, pH 5, at a flow rate of 0.5 ml per minute. Known pyrimidine compounds were added to normal control urine and identified in the eluate by their ultraviolet absorption spectra as determined in a Cary recording spectrophotometer. 6-AZUR was found in fractions 1–3, orotidine in 9–11, and orotic acid in 15–19.

The pattern of elution was determined for each urine sample studied by measurement of the optical density of each fraction at 266 and 279 mμ in a Unicam S. P. 500 spectrophotometer. The quantity of pyrimidine present in each sample was calculated from the optical density at the appropriate wave length of pooled fractions using the following molar extinction coefficients: E_{282} 6-AZUR, 6,100; E_{266} orotidine, 10,000; E_{279} orotic acid, 7,580. A small correction in this value was made for any optical density contributed by other substances present in the urine prior to drug therapy or resulting from the action of formate on the resin bed itself.

Concentrations of these compounds above 5 mg per 100 ml were accurately determined by these methods, although the presence of each could usually be noted in concentrations as low as 2 mg per 100 ml.

Renal clearance studies. All clearance studies were performed on catheterized subjects by the technique of Smith (18). Clearance studies consisted of three 20-minute control periods, three 20 to 30-minute periods during infusions of 6-AZUR and two 20 to 40-minute collections after discontinuing the infusion. Urine was then collected in 4-hour fractions after removal of the catheter and corresponding plasma samples obtained for determination of urate and creatinine clearance.

RESULTS

Uricosuria during 6-AZUR therapy. The uricosuric effect of 6-AZUR observed during therapy of six patients with chronic leukemia on whom daily 24-hour urate and creatinine clearances were obtained is shown in Table I. The urinary excretion of uric acid was increased two- to threefold and the serum urate levels were reduced by 40 to 75 per cent during therapy with 6-AZUR. Figure 2 illustrates the twofold increase in uric acid clearance, the decrease in serum urate levels and the concomitant appearance in the urine of orotic

TABLE II

Relation of uric acid excretion to dose of Azauridine

Dose 6-AZUR(mg/24 hrs)	none	70	700	1400	2800	450
24-hr. urate(mg)	378	350	469	408	675	744
Serum urate(mg %)	6.68	6.57	6.28	6.05	5.57	4.86
*C creatinine(ml/min)	124	117	126	85	136	139
C uric acid(ml/min)	3.9	3.7	5.0	4.6	8.1	10.6
Ratio $\frac{\text{Cua}}{\text{C creat.}}$ X100	3.2	3.2	4.0	5.4	5.9	7.6
Orotic Acid(mg/24 hrs)	0	19	42	33	81	65
Orotidine(mg/24 hrs)	0	0	52	205	299	221

*C = clearance

TABLE III

Clearance studies during the acute infusion of azauridine *

		Clearance periods†	Plasma uric acid	U.V./min	Clearance U.A.	Clearance inulin	$\frac{C_{UA}}{C_{In}} \times 100$	Orotic acid†
			mg %	*mg/min*	*ml/min*	*ml/min*		*mg*
Malignant melanoma F, 42	AZUR 4.6 g	1	3.06	0.587	19.2	145	13.2	0
		2	3.23	0.653	20.2	147	13.7	0
		3	3.38	0.582	17.2	128	13.4	0
		4	3.19	0.538	16.9	119	14.2	0
		5	3.15	0.693	22.0	133	16.5	0
		6	3.28	0.780	23.8	141	16.9	0
		7	3.21	0.769	23.9	131	18.2	0
		8	3.03	0.815	26.9	124	21.7	3
Chronic myelogenous leukemia M, 31	AZUR 4.8 g	1	6.58	0.927	14.09	144	9.75	0
		2	6.87	0.986	14.36	129	11.14	0
		3	6.74	1.211	17.97	150	11.98	0
		4	6.66	1.462	21.95	144	15.17	0
		5	6.32	1.761	27.87	161	17.25	0
		6	6.43	1.796	27.93	151	18.51	0
		7	6.30	1.806	28.67	152	18.85	0
		8	6.28	2.011	32.01	162	19.69	0
Polycythemia vera M, 68	AZUR 5 g	1	10.50	0.656	6.25	85.1	7.3	0
		2	10.98	0.673	6.13	81.7	7.5	0
		3	10.88	0.723	6.65	85.5	7.8	0
		4	11.10	0.805	7.25	88.2	8.2	0
		5	11.14	0.911	8.18	75.8	10.8	0
		6	11.04	1.406	12.74	88.0	14.5	0
		7	11.06	1.474	13.33	77.3	17.2	9
		8	10.66	1.478	13.86	82.0	16.9	17

* The following abbreviations are used: F = female; M = male; U.V. = urine concentration times volume; U.A. = uric acid; C_{UA} = uric acid clearance; and C_{In} = inulin clearance.

† Each period was 20 minutes.

‡ Absence of orotic acid denotes <2.5 mg%; orotidine was absent (<5 mg%) from all urines.

acid and orotidine in quantities as large as 3 g daily as a result of the daily infusion of 4.2 g of 6-AZUR. This patient showed no therapeutic response to treatment with 6-AZUR, and therefore a pronounced dissolution of leukemic tissue in response to chemotherapy probably did not contribute appreciably to his increased urate excretion.

The effects of gradually increased daily doses of 6-AZUR on various parameters of urate excretion are shown in Table II. Infusion of 2,800 mg of 6-AZUR per day produced a definite uricosuria. Lower doses did not cause the urate retention often seen with uricosuric drugs (19).

Clearance studies during 6-AZUR infusion. The magnitude and duration of the effects of 6-AZUR on urate and inulin clearance were simultaneously determined. A prompt increase in uric acid clearance and urate/inulin clearance ratios without alteration in inulin clearance was noted after 6-AZUR infusion (Table III). Orotic acid did not appear in the urine until at least 90 minutes after the start of 6-AZUR infusion and was unaccompanied by orotidine for the duration of the clearance studies.

The time relationship of uricosuric response to the 6-AZUR infusion and to the urinary excretion of orotic acid (Figure 3) suggested that the uricosuria induced by 6-AZUR could be resolved into two distinct components. The prompt initial uricosuric response to 6-AZUR infusion was unassociated with orotic acid excretion, while the maximal uricosuric effect occurred simultaneously with the greatest excretion of orotic acid. These findings led to an investigation of the uricosuric effect of orotic acid.

Uricosuria induced by orotic acid. The intravenous infusion of 1 g of orotic acid during a 12-hour period in two patients was found to result in a two- to-threefold increase in uric acid excretion rate and a 50 per cent decrease in serum urate (Figure 4). The absolute increase in the rate of uric acid excretion (in milligrams per hour) closely paralleled the rate of orotic acid excretion (Figure 4). An infusion of 100 mg of orotic acid in a 12-hour interval produced no uricosuria in a

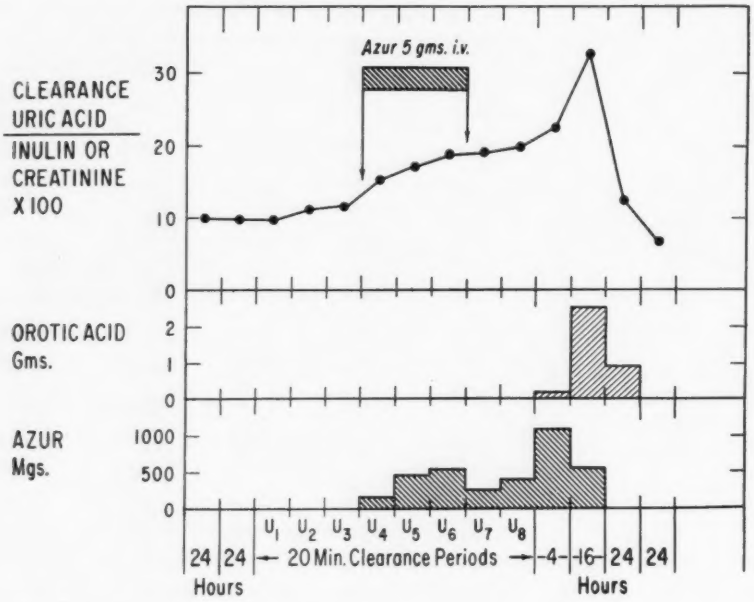

FIG. 3. RELATIONSHIP OF INCREASED URIC ACID CLEARANCE TO THE URINARY EXCRETION OF 6-AZAURIDINE AND OROTIC ACID. Maximal uricosuria occurs simultaneously with maximal orotic aciduria. This patient had polycythemia vera (see Table III).

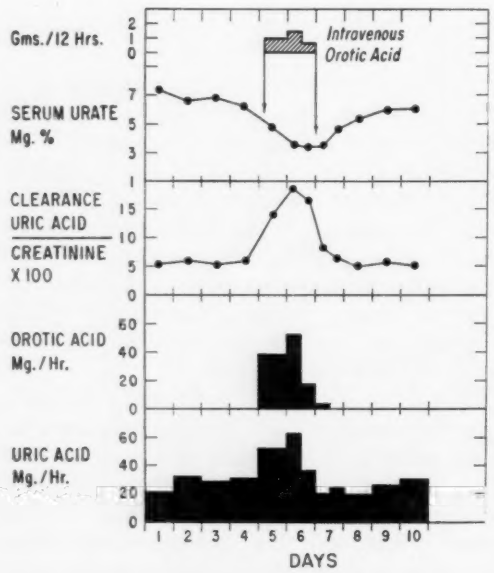

FIG. 4. URICOSURIA PRODUCED BY OROTIC ACID INFUSION. Prompt uricosuria occurs which closely follows, in magnitude, the amount of orotic aciduria.

patient who developed a prompt uricosuria when receiving 2 g per 12 hours.

The urinary recovery of intravenously administered orotic acid was 26 per cent during infusion of 1 g per 12 hours but increased to 48 per cent when the infusion rate was doubled. When orotic acid was given orally in amounts varying from 500 mg to 5 g accompanied by 1 to 2 g sodium bicarbonate to elevate gastric pH, there was no detectable urinary orotic acid and no uricosuria. The intravenous infusion of 660 mg of orotidine to one patient over a 12-hour period produced no increase in urate excretion.

The orotic aciduria and orotidine excretion induced by 6-AZUR. The nature of the major metabolic alteration produced by 6-AZUR was investigated by measuring the urinary excretion pattern of orotic acid and orotidine in patients receiving 6-AZUR. Orotic acid appeared in the urine 90 minutes to 4 hours after the start of 6-AZUR administration, but orotidine did not appear until 1 to 4 hours after the onset of orotic acid excretion (Figure 5).

The largest quantities of orotic acid and orotidine were excreted by patients receiving large amounts of 6-AZUR (120 to 200 mg per kg) and especially by those with chronic myelogenous leukemia. The maximum excretion of orotic acid occurred within 3 days and then declined to a constant level (Figure 2). By contrast the maximal excretion of orotidine appeared later and continued at a high level throughout 6-AZUR administration. Furthermore, orotidine persisted in the urine for 24 to 72 hours after cessation of both 6-AZUR and orotic acid excretion.

There was no observed correlation of orotic acid or orotidine excretion with the antileukemic effects of 6-AZUR. Also, variations in the degree of inhibition of the decarboxylation of orotic acid, as measured by an *in vitro* isotopic assay method (8), in intact leukemic cells, was not well correlated with the magnitude of orotic aciduria.

DISCUSSION

Metabolic alterations. The metabolic alterations resulting from the administration of 6-AZUR to the human are best interpreted as resulting from its inhibition of the decarboxylation of orotidylic acid (Figure 1). Further evidence that this is the site of action of 6-AZUR *in vivo* is the demonstration that intact leukocyte preparations obtained from patients receiving 6-AZUR show a depressed ability to decarboxylate orotic acid (8). *In vitro* studies have shown that 6-AZUR must be phosphorylated by a kinase reaction (requiring ATP and Mg^{++}) in order to function as a competitive inhibitor of this reaction (1, 2). Uridine is in turn a competitive inhibitor of this phosphorylation step *in vitro* (1) as well as an alternate precursor of uridylic acid, and therefore was restricted in the diets of patients receiving 6-AZUR therapy.

Accumulation of orotic acid has been noted in a variety of biological systems. It appears in certain mutant bacteria grown on a minimal pyrimidine media (20), in a rare congenital disorder (orotic aciduria) in man (21), presumably caused by a depressed activity of orotic acid pyrophosphorylase or orotidylic decarboxylase or both (see Figure 1) (22), and as a result of 6-AZUR administration in both bacteria and animals (1, 4, 9, 10). Feedback control by pyrimidine nucleotides of the production of orotic acid has been described in mutant bacteria by Yates and Pardee (23, 24), in Ehrlich ascites cells (25) and in congenital orotic aciduria in the human by Huguley, Bain, Rivers and Scoggins (21).

In mutant bacteria, increased production of aspartate-carbamyltransferase occurs in response to "pyrimidine starvation." The most potent inhibitors of this increased enzyme production are compounds derived from uracil (24), and in addition, cytidine 5′-phosphate is a competitive inhibitor of the same reaction (23). In one case of congenital orotic aciduria the increased orotic acid excretion was significantly depressed by oral administration of a cytidylic-uridylic acid mixture, which suggests a feedback inhibition by pyrimidine nucleotides of orotate synthesis (21) in man.

The finding of increased levels of activity of the first three enzymes of *de novo* pyrimidine biosynthesis (aspartate-carbamyltransferase, dihydroorotase and dihydro-orotic dehydrogenase; see Figure 1) in the white cells of untreated leukemic patients (26) may explain the prompt and massive excretion of orotic acid in leukemic patients receiving 6-AZUR. The possibility also exists that "pyrimidine starvation" (23) induced by 6-AZUR further increases the production of orotic acid by releasing the feedback inhibition which normally regulates pyrimidine biosynthesis. This possibility is currently under investigation.

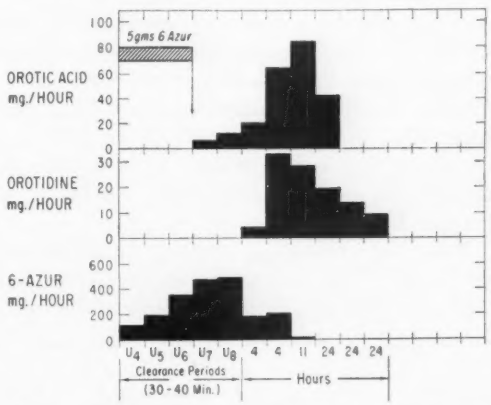

FIG. 5. EXCRETION PATTERN OF OROTIC ACID AND OROTIDINE AFTER 6-AZUR INFUSION. The excretion of orotic acid precedes orotidine and the former disappears more rapidly from the urine. The urinary excretion of 6-AZUR is also noted. The patient had polycythemia vera (see Table III).

Orotidylic acid is the presumed source of the large quantity of orotidine excreted in the urine of patients receiving 6-AZUR, since *in vitro* studies in mammalian tissues, by isotope incorporation methods and assay of the conversion of orotidine to orotic acid, strongly suggest that orotic acid is not a direct precursor of orotidine (1). The consistent appearance in the urine of orotic acid before that of orotidine and the gradually increasing orotidine excretion associated with a decline in orotic acid excretion do suggest, however, that an accumulation of orotic acid may lead to an increase in its conversion to orotidylic acid and then by dephosphorylation to orotidine. In this fashion orotic acid would be an indirect precursor of orotidine by means of its conversion to orotidylic acid. The *in vitro* equilibrium of the conversion of orotic acid to orotidylic acid is strongly in favor of orotic acid formation and, in addition, orotidylic acid is an inhibitor of the enzyme orotidylic acid pyrophosphorylase (27). In order to form large amounts of orotidine, significant production of orotidylic acid is necessary and would be expected to require a large accumulation of orotic acid. Other interpretations of this data exist, however, and the information now available does not prove the primacy of increased *de novo* orotic acid production as an explanation of the pattern of orotic aciduria and orotidine excretion induced by 6-AZUR.

Uricosuria. Increased uric acid excretion is a frequent occurrence during the treatment of leukemia (28, 29) and occasionally results in urate renal blockade. It is usually associated with hyperuricemia and the rapid destruction of leukemic tissue (30, 31). 6-AZUR therapy resulted in a uricosuric response in patients without leukemia or solid tumor as well as in leukemic patients, regardless of clinical response.

The data presented strongly suggest that both 6-AZUR and orotic acid are capable of producing a uricosuric effect. The correlation of maximal uricosuria with maximal orotic aciduria raised the possibility that orotic acid was the sole mediator of the uricosuria seen during 6-AZUR therapy, since orotic acid infusions alone produced increased urate excretion. The prompt uricosuric response to 6-AZUR infusion, which preceded the appearance of orotic acid in the urine, is evidence that 6-AZUR itself has uricosuric properties. Furthermore, infusion of small quantities of orotic acid, which produced detectable amounts in the urine, had no significant uricosuric effect. This finding argues against the possibility that the initial uricosuria induced by 6-AZUR represented a response to quantities of orotic acid which were undetectable by our analytical methods. It is conceivable that the uricosuric action of 6-AZUR could also be mediated by an accumulation of orotic acid within the tubular epithelial cells but there is at present no evidence to support such a concept. In any event it appears that the initial uricosuric effect of 6-AZUR is not dependent on the excretion of orotic acid, and the increased urate clearance seen in patients receiving 6-AZUR results from a summation of the effects of both compounds.

The magnitude of the uricosuric response produced by 6-AZUR or orotic acid infusion resembles that of such moderately active agents as probenecid (32), adrenal steroids (33) and phenylbutazone (34, 35). The administration of each of these compounds usually results in uric acid clearances of 20 to 40 ml per minute in comparison with a normal clearance of 5 to 10 ml per minute. No attempt has been made, however, to produce a maximal uricosuric effect with 6-AZUR or orotic acid because of the potential hazard of orotic acid crystalluria. Neither of these new uricosuric agents has practical value, at the present, in the treatment of hyperuricemia because intravenous administration is necessary for their action. When administered orally, 6-AZUR is partially converted to 6-azauracil (36, 37), a compound which produces severe neurotoxicity in man (38). Oral administration of as much as 5 g per day of orotic acid produced no uricosuria and no orotic acid could be found in the urine.

Urate excretion by the kidney is interpreted by Gutman and Yü (39) to consist of glomerular filtration, nearly complete tubular reabsorption and active tubular secretion. Most uricosuric compounds are acids (zoxazolamine is an exception) (40) and are presumed to act primarly by inhibition of tubular reabsorption of urate. The urate-retaining properties of salicylates and phenylbutazone in low dosage are interpreted as resulting from inhibition of tubular secretion of urate (39). 6-AZUR and orotic acid have not produced this low-dose effect in our studies. 6-AZUR and

orotic acid are both organic acids which are actively secreted by the avian kidney (37, 41) and therefore have some characteristics in common with other uricosuric agents. In addition, two other possible explanations of their mode of action deserve consideration. There are certain structural similarities (Figure 6) (keto groups at corresponding positions on the pyrimidine ring and anionic form at physiological pH) which suggest that these compounds may compete for an enzymatic transfer mechanism that is based on these factors. The observed close quantitative relationship of orotic acid excretion to increases in uric acid excretion may be further evidence that these structural analogs of uric acid may compete for the same transport mechanism involved in urate reabsorption. Evidence has been obtained that urate secretion may also be influenced by the pyrimidine analog, 6-azathymine. We have confirmed the report (42) that this compound produces a urate retention as shown by diminished urate/creatinine clearance ratios.

Although it is possible that the uricosuric effect of 6-AZUR results directly from its inhibition of pyrimidine nucleotide synthesis in the renal tubules, there is at present no evidence to support such a concept. A possible role of uridine nucleotides in phosphate transport by the kidney has been suggested by de Verdier (43). No such role for pyrimidine containing coenzymes has as yet been proposed for other transport systems.

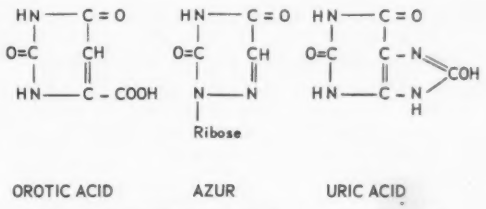

FIG. 6. STRUCTURAL FORMULAS OF OROTIC ACID, 6-AZAURIDINE AND URIC ACID.

SUMMARY

1. Administration of 6-azauridine (6-AZUR) intravenously to patients with nonterminal malignancies resulted in the prompt appearance in their urine of orotic acid and, somewhat later, of orotidine. The appearance of these compounds, also noted by others, provides evidence that 6-AZUR produces the same block in pyrimidine biosynthesis *in vivo* in the human that has previously been noted *in vitro* and in other mammals. The pattern of urinary excretion of these compounds has been discussed with reference to a possible feedback control of pyrimidine biosynthesis in man.

2. The uricosuria induced by 6-AZUR administration resulted in part from the direct action of 6-AZUR and also from the uricosuric action of the orotic acid produced in response to the metabolic block of pyrimidine biosynthesis. The necessity of administering these compounds by the intravenous route prevents their practical use in the management of gout. The chemical structures of 6-AZUR and orotic acid show certain points of similarity to uric acid and they may represent a new class of uricosuric agents based on structural analogs of uric acid. The role of pyrimidines in renal transport mechanisms is, at present, uncertain.

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MEASUREMENT OF ARTERIAL AGING IN HYPERTENSIVE PATIENTS*

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Roach and Burton (1) described two important changes in arterial walls with advancing age. One is an increase in collagenous fibers causing loss of distensibility with increase in the slope dP/dV of arterial pressure-volume curves. The other is a decrease in the unstretched length of these collagenous fibers as the "slack" in them is taken up, possibly by cross linkages and adhesions. This change results in a more rapid decrease in distensibility with stretch and an increase in the rate of change in slope (or the curvature) of pressure-volume curves toward the pressure axis.

An indirect clinical index of the latter effect of aging on arterial walls has been described (2, 3) and referred to, arbitrarily, as an "index of arterial rigidity." It does not measure the slope dP/dV of arterial pressure-volume curves but the rate of change in slope (or the curvature) with pressure. Thus, it is not directly related to distensibility of the large arteries but it represents the differential of their stiffness with respect to pressure.

Conway and Smith (2) applied this test to a study of "aging of arteries in relation to hypertension" and found that the rigidity indices of older hypertensive patients exceeded those of a control group of 16 young medical students. Their observations (2, 4) supported the hypothesis (5) that in the majority of patients with essential hypertension the elevated diastolic pressure is the consequence of aging of arteries. More recently (3) we demonstrated a strong correlation between this index and age which made us suspect that a number of the hypertensives in their studies might be considered to have normal rigidity indices when compared with normal subjects of the same age. Knowing the variation of this index with physiological aging, we could interpret more correctly the results of the test in hypertensive patients of all age groups. This report presents our results in 100 hypertensive patients. The foregoing hypothesis concerning the etiology of hypertension is re-evaluated.

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MATERIAL AND METHOD

The patients for this investigation came from the wards and outpatient clinic of Milwaukee County Hospital. One hundred ambulatory hypertensive subjects, ranging from 18 to 84 years of age, were studied. Their selection was based on the level of arterial blood pressure without knowledge of the etiology, duration or complications of the hypertension. Neither the reason for their hospitalization nor the presence of any associated disease was known to us. All had a diastolic blood pressure of 110 mm Hg or more, but with most patients taking antihypertensive medications, the majority of the blood pressures recorded at the time of the study were only slightly elevated (Table I).

The arterial rigidity index was measured in all the patients. The method required the measurement of intra-arterial blood pressure changes resulting from the inhalation of amyl nitrite before the onset of reflex tachycardia. The ratio of the change in pulse pressure $\times 100$ to the related decrease in diastolic pressure during this period is the index of arterial rigidity (Figure 1). A detailed protocol of the test procedure has been presented (3). A complete physical examination and a careful review of the clinical record followed the performance of the test in each patient. The indices were calculated without access to the clinical findings.

The subjects were separated into two groups: those who had normal indices for their age and those who had prematurely high indices. The upper limit of normal for each age was obtained by adding 2 standard deviations to the regression equation: index $= (2.19 \times \text{age}) - 53.84 \pm 16.16$ SD. This equation was determined from our study (3) of the effect of age on the arterial rigidity index and was effective in separating the rigidity of aging from that of premature arteriosclerosis. Observations on the clinical characteristics of the two groups of patients were made in an attempt to re-evaluate the relation between hypertension and arteriosclerosis. Probability values were obtained by Student's *t*-distribution and the chi-square test (6).

RESULTS

Seventy-one hypertensive patients had normal rigidity indices for their age and 29 had indices that were higher than normal (Figure 2). Table I shows some clinical characteristics of each patient.

Seventy-one hypertensive subjects with normal rigidity indices. The average age of this group

TABLE I

Individual clinical observations on 100 hypertensive patients classified according to their arterial rigidity indices *

Case no.	Age	Race and sex	Resting intra-arterial pressure	Type of hypertension	Duration of hypertension		Diabetes mellitus	Retinal changes (grade I to IV)	Family history of		Left ventricular hypertrophy	Aortic tortuosity or calcification	Arterial rigidity index†
					<10 yrs	>10 yrs			Diabetes	Hypertension			
	yrs		*mm Hg*										
					Patients with normal rigidity indices								
1	23	NF	120/79	Renal	+			I					10
2	29	WM	155/97	Labile	+								0
3	31	NF	186/110	Fixed	+								33
4	33	NM	130/85	Labile	+			I					−20
5	34	NM	218/120	Labile	+			I					45
6	34	WF	160/95	Fixed	Unknown			II					4
7	34	WM	148/89	Renal (K.W.)	Unknown		+	II		+			0
8	35	WF	159/104	Labile	+			I					−10
9	35	WM	170/90	Fixed	+			II					50
10	36	NM	150/90	Fixed	Unknown								25
11	36	NF	155/95	Fixed	+			II	+				50
12	37	NM	150/90	Fixed	+			I	+				8
13	37	NF	200/115	Fixed	Unknown			II	+	+	+		43
14	38	NF	205/125	Fixed	+			II		+	+		33
15	39	NM	255/150	Renal	+			IV		+	+		7
16	40	NF	163/116	Fixed		+		II		+	+	+	25
17	40	NF	244/120	Renal	+			III			+	+	45
18	40	WM	130/77	Labile	Unknown								39
19	42	WM	165/109	Labile	+			I	+	+			16
20	43	NF	192/105	Fixed	+			III					40
21	43	NF	135/83	Fixed	Unknown			II	+	+	+		36
22	43	WF	180/110	Renal	Unknown			III			+		40
23	43	NM	135/85	Fixed	Unknown								40
24	44	NF	200/110	Fixed	+			III			+		50
25	45	WF	175/95	Fixed	+			II					73
26	46	NM	210/125	Fixed	Unknown			IV			+		0
27	46	NM	140/70	Fixed		+		II	+	+	+		74
28	47	WM	185/105	Labile	Unknown								27
29	47	WM	127/70	Fixed	+			II					63
30	48	NM	220/100	Fixed	Unknown			II		+	+		45
31	49	WM	230/110	Renal	Unknown			III		+	+		30
32	49	WM	154/95	Renal		+		II	+		+		35
33	49	NM	185/115	Fixed	+								30
34	49	WF	233/125	Fixed		+		III		+	+		40
35	50	WF	150/90	Fixed	Unknown								60
36	50	WF	205/115	Renal	+			I					47
37	50	WM	262/142	Fixed	+			II		+	+		66
38	51	WM	145/80	Fixed	Unknown		+						75
39	51	WF	185/103	Labile	Unknown		+						84
40	52	WM	160/80	Fixed	+			I					35
41	52	NF	160/110	Fixed	+		+		+				47
42	53	WM	170/100	Fixed	+			I					33
43	53	WF	215/103	Fixed	Unknown		+	I					75
44	53	NF	170/98	Fixed	+			I					60
45	55	WF	130/70	Undetermined	+		+						47
46	55	NM	175/115	Renal	+			II			+		35
47	55	WM	135/84	Labile	Unknown				+			+	37
48	56	NF	235/125	Fixed	+			II	+	+	+		75
49	56	NM	200/115	Fixed	+								91
50	56	WM	175/85	Fixed	Unknown			I	+				67
51	57	WF	150/80	Fixed	Unknown								100
52	58	WM	190/95	Fixed	Unknown			II	+				100
53	58	NF	240/101	Fixed		+		II			+		100
54	60	NF	270/170	Fixed	Unknown			III			+	+	77
55	60	WF	180/85	Fixed	Unknown								60
56	60	WM	170/90	Fixed	Unknown			II					73
57	60	WM	150/88	Fixed		+		II	+	+	+		81
58	61	WF	202/105	Undetermined	+								50
59	61	WM	169/93	Fixed	+			II			+		62
60	64	NM	233/98	Fixed		+		II	+		+	+	54
61	64	WF	185/90	Renal	+								95
62	64	WM	190/110	Renal	Unknown			II				+	100
63	64	WM	270/105	Fixed	Unknown			II				+	100
64	65	WM	200/105	Fixed	Unknown								50
65	65	WF	155/70	Fixed	Unknown				+	+			75
66	65	WM	212/112	Fixed	+			II					70
67	70	WM	220/120	Fixed	Unknown			III				+	127
68	71	WF	278/101	Renal	+			III		+	+		89
69	72	WF	194/99	Undetermined	+					+			76
70	74	WM	206/115	Fixed	Unknown			II	+				48
71	81	WF	155/69	Fixed	Unknown			I				+	122

* Abbreviations: N =negro; W =white; M =male; F =female; K.W. =Kimmelstiel-Wilson's syndrome.
† The upper limit of normal at each age is shown in Figure 2.

TABLE I—(*Continued*)

Case no.	Age	Race and sex	Resting intra-arterial pressure	Type of hypertension	Duration of hypertension		Diabetes mellitus	Retinal changes (grade I to IV)	Family history of		Left ventricular hypertrophy	Aortic tortuosity or calcification	Arterial rigidity index†
					<10 yrs	>10 yrs			Diabetes	Hypertension			
	yrs		*mm Hg*										
					Patients with prematurely elevated indices								
1	18	WF	160/110	Labile	+		+						42
2	29	WF	141/81	Fixed	+			I	+				78
3	30	NM	154/83	Renal (K.W.)	+		+	II			+		53
4	31	WF	175/115	Labile	+								84
5	41	WF	233/119	Fixed	+			III	+	+	+		127
6	41	WM	195/100	Renal (K.W.)	Unknown		+	II	+				82
7	44	WM	152/90	Renal	Unknown		+	II				+	83
8	46	NF	190/95	Fixed	Unknown		+	II			+	+	100
9	46	WM	190/95	Renal (K.W.)		+	+	III			+	+	82
10	46	WF	155/90	Labile	Unknown								100
11	46	WM	210/90	Fixed	+			III	+	+	+		105
12	48	WF	265/110	Renal (K.W.)		+	+	III	+		+		136
13	48	WF	176/86	Undetermined	Unknown			I			+		118
14	49	WM	190/100	Fixed		+		III			+		105
15	50	WF	218/108	Fixed	Unknown		+	II			+		108
16	50	WM	141/53	Fixed	Unknown			III					120
17	52	WF	118/55	Fixed		+		II	+	+		+	100
18	52	WF	180/85	Fixed		+		II					105
19	55	WF	200/70	Renal	+		+	III				+	130
20	56	WF	191/86	Fixed	Unknown			II					135
21	58	WF	195/90	Fixed		+	+	II			+	+	143
22	59	WF	200/110	Fixed		+		III			+	+	180
23	59	WF	180/80	Renal (K.W.)		+	+	IV	+		+	+	200
24	63	WM	240/120	Fixed		+		IV		+			160
25	70	WF	220/90	Renal		+		II					173
26	80	WM	210/75	Fixed	Unknown			II					168
27	83	WF	215/85	Fixed	Unknown			II				+	200
28	84	WF	180/50	Fixed	Unknown			II				+	200
29	84	WF	210/80	Fixed		+		II				+	175

was 49.8 ± 14.0 (SD) years. Thirty-three (46.4 per cent) patients were females; 17 had a family history of hypertension and 16 a family history of diabetes. Six patients had diabetes mellitus. Of 41 in whom the duration of hypertension could be determined, only 7 had had it for more than 10 years. The type of hypertension was diagnosed as labile in 9, fixed essential in 47, renal in 12 (1

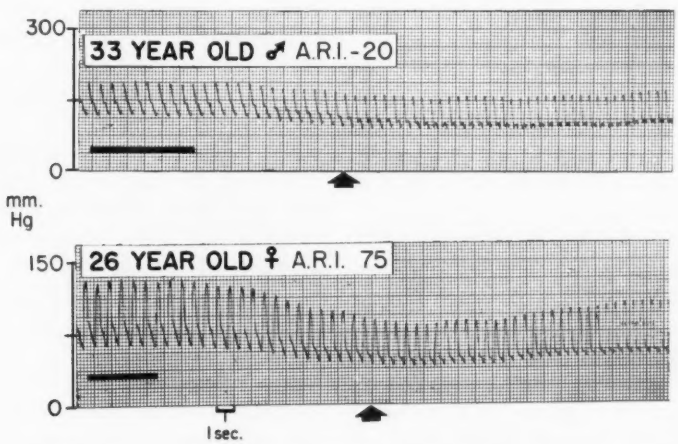

FIG. 1. BRACHIAL ARTERIAL PRESSURES RECORDED BEFORE, DURING AND AFTER AMYL NITRITE INHALATION IN 2 SUBJECTS. The horizontal lines below the tracings indicate the time of inhalation of amyl nitrite. The vertical arrows indicate the onset of reflex tachycardia. When the change in pulse pressure is negligible (upper tracing) the arterial rigidity index (A.R.I.) is small (−20). When the fall in pulse pressure is marked (lower tracing) the A.R.I. is high (+75).

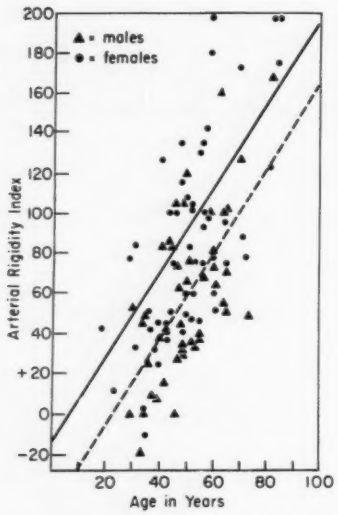

FIG. 2. ARTERIAL RIGIDITY INDICES (ORDINATE) OF 100 HYPERTENSIVE PATIENTS RANGING FROM 18 TO 84 YEARS OF AGE. The interrupted line represents the regression of this index on age in normal subjects; the solid line is the upper limit of normal (3). Seventy-one hypertensive patients had indices below the upper limit and 29 had prematurely elevated indices. The incidence of hypertensive females with premature arterial aging was high.

had Kimmelstiel-Wilson's syndrome), and undetermined in 3. The average diastolic pressure at the time of the test was 101.0 ± 22.0 mm Hg and the average pulse pressure was 82.9 ± 27.0 mm Hg. There was roentgenographic evidence of tortuosity and calcification of the aorta in 9 patients, 6 of whom were over 60 years of age. Electrocardiographic evidence of left ventricular hypertrophy was present in 23 and grade III or IV eye-ground changes were found in 11.

Twenty-nine hypertensive subjects with prematurely high rigidity indices. The average age of this group was 52.4 ± 16.5 (SD) years. Twenty (68.9 per cent) patients were females; 4 had a family history of hypertension and 7 had a family history of diabetes. Eleven patients had diabetes mellitus. Eleven of 18 in whom the duration of hypertension could be determined had been hypertensive for more than 10 years. There were 3 patients with labile hypertension, 17 with fixed hypertension and 8 with renal hypertension, 5 of whom were diabetics with Kimmelstiel-Wilson's syndrome. In one subject the type of hypertension had not been determined. The average diastolic pressure of this group was 89.7 ± 18.3 mm Hg and the average pulse pressure was 99.4 ± 26.7 mm Hg. Tortuosity or calcification of the aorta was present in 11, 8 of whom were younger than 60 years of age. There was electrocardiographic evidence of left ventricular hypertrophy in 12, and grade III or IV hypertensive retinopathy was found in 10.

Comparison between the two groups. As shown in Table II, there were relatively more diabetics and patients with hypertension of more than 10 years' duration in the group with prematurely elevated indices. The incidence of females was higher also in this group and grade III or IV eye-ground changes were more common as were other abnormalities seen with advanced arteriosclerosis, such as a wider pulse pressure, lower diastolic pressure and tortuosity of the aorta. There was no difference between the two groups in regard to age, type of hypertension, incidence of left ventricular hypertrophy and incidence of family history of hypertension or diabetes.

DISCUSSION

The results show that only 29 hypertensive patients had high rigidity indices for their own age and 71 had normal indices. It is apparent that premature aging of the arteries of hypertensive patients is not nearly as prevalent as was reported (2, 4). The main reason for this discrepancy lies in the interpretation of the results. Although the old hypertensive patients in our study had high rigidity indices, many were considered "normal" in view of the progressive increase of the index with age which we had demonstrated (3). This effect of physiological aging was not considered by others (2, 4) who reported that the majority of hypertensive patients had abnormally rigid arteries and postulated that the processes leading to rigidity of the large arteries were also associated with sclerosis of the peripheral vascular bed and diastolic hypertension. Our findings do not support this hypothesis, since the majority of patients in this study had normal rigidity indices for their age.

These results indicate that the hypertensive patients have no greater decrease in distensibility with stretch than do the normals with age, although they might indeed have less distensible ar-

TABLE II

Comparison of clinical observations made on normotensive subjects, hypertensive patients with "normal rigidity indices" and hypertensive patients with "prematurely elevated indices"

	Normotensive subjects* (A)	Hypertensive patients with		Significance of difference (p values) between	
		Normal indices (B)	Prematurely elevated indices (C)	A and B	B and C
No. of patients	115	71	29		
Average age (*yrs*)	52.8	49.8	52.4	>0.2	>0.4
Sex, females	42	33	20	>0.1	<0.05
Family history of					
Diabetes (%)		22.5	24.1		>0.5
Hypertension (%)	17.3	24.0	13.8	>0.2	>0.1
Incidence of diabetics (%)		8.5	38.0		<0.001
Duration of hypertension					
>10 yrs (%)		9.8	38.0		<0.001
<10 yrs (%)		47.9	24.0		>0.05
Undetermined (%)		42.3	38.0		>0.7
Average arterial pressures (*mm Hg*)					
Systolic	134.8	183.9	189.1	<0.001	>0.1
Diastolic	69.8	101.0	89.7	<0.001	<0.01
Pulse pressure	65.0	82.9	99.4	<0.001	<0.01
Type of hypertension					
Labile (%)		12.6	10.3		>0.7
Fixed (%)		66.2	58.8		>0.4
Renal (%)		16.9	27.5		>0.5
Undetermined (%)		4.3	3.4		>0.8
Retinopathy (grade III-IV) (%)		15.5	34.5		<0.05
Left ventricular hypertrophy (%)		32.4	41.3		>0.1
Tortuosity or calcification of aorta (%)	5.2	12.6	38.0	>0.05	<0.01

* These observations were made in a previous study (3) in which the effect of age on the rigidity index was determined. These "normal" subjects were selected to satisfy certain criteria among which were the absence of a family history of diabetes, of grade III or IV retinopathy and of left ventricular hypertrophy.

teries. The pulse pressure and not the "rigidity index" would be the indication of distensibility, although it would have to be considered at comparable mean arterial blood pressures. Adamson and Doupe (7) showed that the central pulse wave velocity was increased in hypertensive patients, but only to the same extent as it would be in normals if their aortic mean pressure were equally raised; i.e., there was no indication of increased rigidity of the aorta in hypertension except in relation to the raised pressure. They concluded that defective arterial elasticity was not a factor in the pathogenesis of diastolic hypertension.

Although distensibility generally decreases with a rise in arterial pressure (8), the "rigidity index" probably remains constant over a fairly wide range of pressures (170 to 55 mm Hg) (3) beyond which it may either increase or decrease. This is because the index is directly related to the "curvature" and not the slope of arterial pressure-volume curves. However, when arterial mean pressure approximates 200 mm Hg the distensibility curves of younger aortas bend more and more toward the pressure axis (9) and the index tends to increase, while the curves of older vessels lose their curvature and the index tends to decrease. The

average mean pressures[1] of the two groups of hypertensive patients at the time of the test were comparable (128.6 and 122.8 mm Hg), well below 200 mm Hg and not very much higher than the average mean pressure of the normotensive group (91.5 mm Hg). This made it possible to compare the indices of the hypertensive patients with those of "normal" subjects of the same age. Only three patients (nos. 15, 37 and 54) had diastolic pressures higher than 125 mm Hg, and consequently their low rigidity indices might have been caused by their high arterial mean pressures, which were over 180 mm Hg in two and over 200 mm Hg in the third.

The clinical features of the patients were examined carefully in an attempt to clarify the relation between hypertension and arteriosclerosis. Two observations were felt to be worthy of emphasis. The first was the higher incidence of diabetics in the group with premature arterial aging ($p < 0.001$). Diabetes has been shown to be associated with, or even preceded by, extensive vascular changes (10–13), while in essential hypertension, biopsy studies of various organs have not generally revealed morphological changes in the peripheral vessels (14, 15). It would seem more reasonable to attribute the premature arterial aging in the 11 diabetics in this group to their diabetic state rather than to their hypertension. A second important observation was the greater incidence of patients who had had hypertension for more than 10 years among those with prematurely elevated indices ($p < 0.001$). This relationship between the known duration of hypertension and aging of arteries suggests that the development of sclerotic vascular changes is a consequence rather than a cause of hypertension.

Autopsy studies have shown an association of hypertension with premature arteriosclerosis primarily in women (16). In this report, 20 of the 29 hypertensive patients with premature arterial aging were females. Master (17) has pointed out that three-fourths of females and only one-fourth of males with coronary occlusion had pre-existing hypertension. Why hypertension should be a prerequisite for the majority of females and only a minority of men who succumb to coronary occlusion is not clear. Whatever the reason, the available evidence seems to favor the hypothesis that hypertension and arteriosclerosis, although interrelated, are two separate entities, their progress being influenced by independent factors.

Of the 29 hypertensive patients in the group with high indices, 7 had diabetes, 7 had had hypertension for more than 10 years, 4 had had both diabetes and hypertension for more than 10 years, 3 were over 80 years of age, 3 had a strong family history of diabetes and 1 had myxedema. Thus in 25 patients, premature arterial aging might have developed either as a complication of hypertension or in association with another disease independent of hypertension. In only 4 of the 100 hypertensive patients was there unexplained premature arterial aging.

[1] Mean arterial pressures were obtained by adding one-third of the pulse pressure to the diastolic pressure.

SUMMARY

The purpose of this study was to re-examine the relationship between hypertension and aging of the arteries by means of the amyl nitrite test of arterial rigidity.

Our findings in 100 hypertensive patients revealed no evidence of premature arterial aging in 71 of them when they were compared with normal subjects of the same age.

The clinical features of the 29 hypertensive patients with abnormal rigidity indices suggested that arteriosclerosis and hypertension developed as two separate entities possibly potentiating each other. We found no evidence to suggest that hypertension might have been caused by arteriosclerosis extending to the peripheral vascular system. If structural changes in the peripheral vascular bed play a major role in the etiology of essential hypertension, they do not appear to be related to premature arterial aging as determined by this test.

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SODIUM AND POTASSIUM IN THE WALLS OF ARTERIOLES IN EXPERIMENTAL RENAL HYPERTENSION *

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In previous studies (1–6) it has been determined that the aortic wall of rats with various forms of experimental hypertension contains an increased amount of sodium, potassium and water per unit of dry weight. The pertinence of these findings to hypertension has always been somewhat in question, since the aorta does not contribute much to the total peripheral resistance. In the normal circulation most of the peripheral resistance is provided by the arterioles, and hypertension is accompanied by a narrowing of these arterioles. Hence an analysis of the arterioles themselves would be expected to give more pertinent information concerning the pathogenesis of hypertension. This was attempted in the present experiment.

The present study was also designed to offset another possible cause of misinterpretation. When an animal becomes hypertensive, the walls of the arterioles become thicker; some of this thickening is probably related to an increased content of acid mucopolysaccharides, which are known to bind sodium (7). In the present experiment a large number of rats was made hypertensive and thickening of the arterioles occurred in all of them. Half the hypertensive rats were then "cured" of their elevated blood pressure by removing the clip which narrowed their renal artery. We were then able to compare the arterioles of hypertensive and normotensive rats, both of which had comparable degrees of arteriolar thickening.

Thus, the present experiment largely overcomes two weaknesses of previous studies. First, electrolytes are estimated in arterioles rather than arteries. Second, there is a similar degree of hypertrophy in both normotensive and hypertensive arterioles.

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METHODS

A number of Wistar rats was made hypertensive by narrowing one renal artery with a clip and removing the opposite kidney. Seven months after the original operation, the rats with moderate to severe hypertension were culled out and divided into two groups which were evenly matched for arterial pressure. In one of the two groups, an operation was performed on each rat to remove the clip which narrowed the renal artery. On the rats of the other group, a similar sham operation was done but the clip was left intact. Exactly 7 days after either type of operation, the blood pressure was carefully determined on each rat with the microphonic method (8), and a number of mesenteric arterioles was removed for analysis with the aid of a stereomicroscope. The arterioles of completely normal rats of the same age were also analyzed concomitantly. The arterioles were handled in such a way that the lumen was free of blood before excision of the vessel. As little adventitia as possible was included in the tissue sample. The arterioles in the samples varied in outer diameter from about 25 to 140 μ. By measuring serial cross sections of the arterioles, it was possible to compute the fraction of the total arteriolar sample which was contributed by arteriolar segments of various sizes (Table I). Arteriolar segments with a lumen diameter varying between 24 and 84 μ contributed 92.5 per cent of the whole arteriolar sample. Maximow and Bloom consider any segment of an artery with an outer diameter of less than 300 μ as an "arteriole" (9). According to this definition, our entire sample is composed of arterioles. Vessels of this size are small enough to contribute importantly to total peripheral resistance.

The sample of arterioles from each rat was dried *in vacuo* at room temperature and then weighed on a microbalance. The dried samples were extracted for a week in Vycor vessels with 0.1 N nitric acid. At the end of this period the extract was filtered and analyzed for sodium and potassium with a flame photometer and for chloride with an amperometric method (10). Utilizing volumes of fluid and concentrations of sodium that were similar to our unknown extracts, we had an average analytical error of 0.59 per cent with 15 samples of a sodium solution and a maximal error of 1.4 per cent. With 15 samples of a potassium solution we had an average error of 0.67 per cent and a maximal analytical error of 3.0 per cent. With 15 replicates of a chloride

TABLE I

Contribution of various sizes of arteriolar segments to the total arteriolar sample

Various groups of arteriolar segments classified according to the inner diameter of the lumen	Average inner diameter of the group	Average outer diameter of the group	Percentage of the total arteriolar sample contributed by the group
μ	μ	μ	
4–12	8	25	0.1
13–24	19	47	2.0
25–36	30	66	9.0
37–48	41	90	17.9
49–60	54	108	26.0
61–72	66	126	25.9
73–84	78	130	13.7
85–96	89	140	5.4

solution, also similar to our unknown solutions in volume and concentration, we had an average error of 2.0 per cent and a maximal error of 4.8 per cent.

During the dissection it was apparent that the arterioles of both the actively hypertensive and previously hypertensive rats were thicker than those of normal rats. The arterioles were always dissected as uniformly as possible, so that the total length of the arterioles in a given sample was about the same for each rat. Since the length of vessels in a sample was approximately constant, the total dry weight of the arteriolar sample gives a reflection of the amount of arteriolar thickening. Table II shows the average of the total dry weights of the samples for the three different groups. The samples from both the continuously hypertensive group and the previously hypertensive group weighed about 53 per cent more than those of the normal group ($p < 0.00001$). However, the samples from the group with continuing hypertension had virtually the same increase in weight as the samples from the group with "cured" hypertension ($p > 0.5$). Thus the arterioles of the rats with

TABLE II

Average dry weight of arteriolar samples from the various groups of rats **

	mg
25 normal rats	2.83 ± 0.16
30 rats with "cured" hypertension	4.27 ± 0.21
25 rats with continuing hypertension	4.37 ± 0.27

* ± Indicates standard error of the mean.

"cured" hypertension seemed to be just as thick as those of the rats with continuing hypertension.

RESULTS

The results of the analyses are shown in Table III. The rats with continuing hypertension had a 16 per cent greater content of sodium in their arterioles than those with "cured" hypertension, a highly significant difference ($p < 0.0001$). The hypertensive rats also had 2.6 per cent more potassium in their arterioles than the rats with "cured" hypertension. This difference was not significant. The chloride content of the arterioles in the group with continuing hypertension was 9 per cent greater than that of the group with "cured" hypertension. The p value of this difference was 0.08, and therefore of borderline significance.

The rats with "cured" hypertension had a slightly but significantly higher mean blood pressure than the completely normal rats ($p = 0.02$; see Table III), hence they were probably not completely "cured." Moreover, they had a definite thickening of the arterioles (Table II). A combination of these two factors probably accounts

TABLE III

Average electrolyte levels in the arteriolar wall of various normotensive and hypertensive rats *

	25 Normal rats (A)	p Value of the difference between A and B	30 Rats with "cured" hypertension (B)	p Value of the difference between B and C	25 Rats with continuing hypertension (C)
Na (*mEq/100 g dry tissue*)	22.1 ± 0.4	0.03	23.5 ± 0.5	0.00006	27.3 ± 0.7
K (*mEq/100 g dry tissue*)	21.2 ± 0.3	>0.5	21.2 ± 0.3	0.3	21.7 ± 0.4
Cl (*mEq/100 g dry tissue*)	21.5 ± 0.8	0.5	22.3 ± 0.7	0.08	24.3 ± 0.9
Na + K (*mEq/100 g dry tissue*)	43.3 ± 0.6	0.14	44.6 ± 0.7	0.0004	49.0 ± 1.0
Arterial pressure† before clip removal (*mm Hg*)	126 (101–140)		197 (160–236)		202 (158–250)
Arterial pressure† one week after clip removal or sham operation (*mm Hg*)	125 (100–141)	0.02	132 (111–152)		197 (157–245)

* ± Indicates the standard error of the mean.
† Mean and range.

for the increased amount of sodium and chloride in the arterioles of rats with "cured" hypertension when compared with those of completely normal rats. The potassium in the arterioles was the same for these two groups.

The concentrations of sodium, potassium and chloride in the serum are given in Table IV. These concentrations are not significantly different in any of the three groups.

TABLE IV

*Mean concentrations of sodium, potassium and chloride in the serum of various normotensive and hypertensive rats**

	25 Normal rats	30 Rats with "cured" hypertension	25 Rats with continuing hypertension
Sodium	142.4	142.5	142.7
Potassium	3.6	3.7	3.7
Chloride	104.8	104.8	105.1

* Concentrations are in mEq/L. The p value of the differences between the groups was in every case greater than 0.3.

DISCUSSION

Although the rats with continuing hypertension have more sodium in their arterioles, it is hard to be sure what fraction of it is intracellular and what fraction is extracellular. The chloride content in the arterioles of the hypertensive rats is also increased about 2 mEq per 100 g of dry arteriole. If one assumes that all of this increment of chloride is extracellular, it should be accompanied by 2.45 mEq of extracellular sodium per 100 g of dry arteriole. This calculation, allowing for Donnan factors, assumes that the concentration of interstitial sodium is 0.95 that of serum sodium and that the concentration of interstitial chloride is 1/0.95 that of the serum chloride (11). The actual difference in the sodium content of the arterioles from the hypertensive group compared with the "cured" group was 3.79 mEq per 100 g. If only 2.45 mEq per 100 g were extracellular, this would then leave 1.34 mEq per 100 g that is intracellular. Hence, with these assumptions concerning chloride, the hypertensive arteriole would have a definite increase of intracellular sodium. Furthermore, one has no real basis for assuming that the increment of chloride in the hypertensive arterioles is indeed all extracellular. Such an assumption might be on somewhat firmer ground with regard to skeletal muscle. However, a recent investigation of the smooth muscle cells in the taenia coli of guinea pigs indicates that there are 74 mEq of chloride and 73 mEq of sodium per L intracellular water (12). The smooth muscle cells in the walls of arterioles may also normally contain an appreciable amount of intracellular chloride and may contain even more in the presence of hypertension. Hence, the data presented here would indicate that at least some of the increment of sodium in the hypertensive arterioles is located intracellularly, and it is possible that almost all of it is intracellular.

It is of interest to compare the present data with those previously obtained using the wall of the aorta. In several forms of experimental hypertension, both sodium and potassium were significantly elevated in the aortic wall (2–6). In the present study the sodium content was significantly increased but potassium was not. In the previous studies on the rat aorta, the chloride content was either not increased at all or increased only slightly and to a lesser degree than the increase in sodium (2–6). The present results would agree better with the latter pattern of change.

Recent studies have indicated that intracellular osmolality is usually similar to that in the extracellular compartment (13). In previous studies with the wall of the rat aorta or the human renal artery, the total cation content ($Na + K$) strongly correlated with the total water content (1, 3). In the present study (Table III) the total cation content ($Na + K$) is 10 per cent greater in the arterioles from hypertensive rats compared with the rats with "cured" hypertension ($p = 0.0004$). The total cation content is 13 per cent greater in the hypertensive arterioles compared with the arterioles from completely normal rats ($p < 0.00001$). If the total cation content has its usual relationship to tissue water in hypertensive arterioles, the data would suggest that the water content per unit of solids is about 10 per cent greater in these hypertensive arterioles than in the normotensive ones. However, such an assumption cannot be made with absolute safety. If, indeed, there were an increase of water in relation to solids in the walls of the hypertensive arterioles, the extra water could encroach somewhat on the lumen and increase resistance to flow (1). Further speculations concerning the cations and water in arterial wall have been recently considered elsewhere (14).

We allowed our rats with "cured" hypertension only 7 days to achieve their cure. Admittedly, their blood pressure was almost down to normal by this time, but it was slightly elevated in about 25 per cent of the rats with "cured" hypertension. Moreover, Floyer has shown that rats with a clip removed from the renal artery retain some evidence of their hypertensive diathesis for 1 to 2 weeks, even though their blood pressure comes down to near normal in a few hours (15). If the lone remaining kidney in his rats was excised 1 to 2 weeks after the clip had been detached from its renal artery, these rats developed renoprival hypertension at a decidedly accelerated rate, indicating that some hypertensive trait lingered on. In the present study, we are comparing hypertensive rats with rats whose hypertensive diathesis is only partially "cured." The difference in electrolyte content might have been even greater if the comparison had been made with rats that were given enough time after removal of the clip to achieve a complete "cure." This lack of a complete cure in the present experiment may partially account for the differences between the rats with "cured" hypertension and the rats that were completely normal.

SUMMARY

Fifty-five rats were made hypertensive by narrowing the renal artery of their one remaining kidney. Seven months later these hypertensive rats were divided into two evenly matched groups. The rats in one group were "cured" of their hypertension by releasing the arterial constriction. The rats in the other group had a comparable sham operation. One week after either procedure, a sample of arteriolar tissue from vessels of 0.025 to 0.14 mm outer diameter was obtained. Arterioles from the rats with continuing hypertension had 16 per cent more sodium per unit of dry weight than those from the rats "cured" of hypertension ($p < 0.0001$). The potassium in the arteriolar wall was similar in the two groups and a difference in chloride content was of borderline significance ($p = 0.08$). At least part of the increment of sodium in the hypertensive arterioles appears to be located intracellularly. A 10 per cent increase in the total cation content ($Na + K$) in the hypertensive arterioles ($p = 0.0004$) may indicate that these vessels have about 10 per cent more water per unit of dry weight than the arterioles from the rats with "cured" hypertension. The arterioles from the rats with either continuing or "cured" hypertension had undergone about the same degree of thickening; hence the difference in sodium content is probably not related to this. The extra sodium in a hypertensive arteriole may contribute to its narrowed lumen.

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THE PLASMA DISAPPEARANCE TIME AND CATABOLIC HALF-LIFE OF I^{131}-LABELED NORMAL HUMAN GAMMA GLOBULIN IN AMYLOIDOSIS AND IN RHEUMATOID ARTHRITIS *

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In 1906, Lewis (1) suggested the possibility that an abnormality in globulin metabolism might play a role in the pathogenesis of amyloidosis because he observed that hyperglobulinemia regularly occurred in horses that developed amyloid as a result of tetanus toxin administration. A similar association between prolonged hyperglobulinemia and the ultimate development of amyloidosis has been noted in a variety of laboratory animals (2–4) and in human beings having either the primary form of the disease (5) or that secondary to rheumatoid arthritis or other chronic inflammatory conditions (6). Whether this association is merely coincidental or of pathogenic significance is not yet known.

While no one globulin fraction has been found to be elevated in all instances and types of amyloidosis, γ-globulin is frequently increased early in the course of the disease and its concentration may decrease during the period of rapid accumulation of amyloid (7). Vasquez and Dixon (8) have found that amyloid, both in humans and in animals with experimental disease, contains γ-globulin which may be revealed by the Coons fluorescein-labeled antiglobulin technique. On the other hand, studies by Calkins, Cohen and Gitlin (9), using an immunochemical technique, have failed to demonstrate γ-globulin in amyloid. Because of these conflicting observations, it was felt that a study of the fate of intravenously injected, labeled γ-globulin in subjects with amyloidosis might throw further light on the relationship between circulating γ-globulin and amyloid. A group of patients with active rheumatoid arthritis was also studied, with the assumption that an illness known to predispose to the development of amyloidosis might reveal a similar alteration in γ-globulin metabolism.

MATERIALS AND METHODS

In the present study, the rate of disappearance from the circulation of intravenously administered I^{131}-labeled γ-globulin, its catabolic half-life, and distribution space were determined. Subjects studied included 4 patients with rheumatoid arthritis, 3 with amyloidosis and 3 normal controls. One of the patients with amyloidosis was first studied as an arthritic. After the first study he developed manifestations of amyloidosis and was reinvestigated 1 year later. Brief case summaries of these patients are appended. The laboratory data are given in Table I.

Labeling of the γ-globulin with I^{131} was carried out by the method of Gitlin, Janeway and Farr (10), a modification of that of Pressman and Eisen (11). The protein employed was poliomyelitis immune globulin prepared by Cutter Laboratories. Four lots of labeled protein were prepared in the course of the study. Although the specific activity and the protein concentration of the material varied from lot to lot (see Table II), the amount of total iodine relative to protein was calculated in such a way that a maximal labeling of 3 atoms of iodine per molecule of protein was anticipated. After dialysis against saline, the protein solution contained less than 1 per cent nonprotein-bound iodine. No iodine was released from the protein after storage of the solution at 4° C for 30 days. Paper electrophoresis of the protein after storage and radiography using photographic film, revealed a homogeneous material containing radioactivity only in the γ-globulin band.

Each subject studied received 24 to 60 μc of radioactive iodine contained in about 20 mg of γ-globulin. After an intravenous infusion of the protein, serum samples were obtained daily for the first 2 weeks and on alternate days thereafter for a total period of 1 month. In addition, during the first 2 days of the experiment, serum was obtained at more frequent intervals, as may be seen in Figure 1. The total amount of blood withdrawn from each

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TABLE I

Pertinent laboratory data for subjects used in this study

Subjects	Age	Duration of disease	Involvement	Associated disease	Total protein	Albumin	γ-globulin*	NPN
	yrs	*yrs*			*g %*	*g %*	*g %*	*mg %*
1. Amyloidosis								
G.P.	55	2	Skin, heart	None	6.8	3.5	0.5	20
N.S.	38	1	Kidney, liver	Rheum. arthritis	8.8	2.9	3.1	23
M.F.	50	1	Kidney	None	3.1	0.6	0.5	23
2. Rheum. arthritis								
B.S.	60	34	Hands, feet spine	None	7.8	3.6	1.3	28
J.P.	61	9	All joints	None	9.8	4.9	2.1	29
L.S.	62	8	Hands, feet spine	Diabetes mellitus	8.2	2.6	3.0	33
3. Normal								
F.T.	63				6.6	4.5	2.1	29
J.G.	48			Old poliomyelitis	6.4	4.4	1.8	
F.W.	41				7.0	4.8	2.0	27

* Calculated from paper electrophoresis.

subject over 1 month was about 150 ml or 3 per cent of the total blood volume. Serial 24-hour urine collections were made for as long as radioactivity could be detected. From the pooled urine specimen for each 24-hour period, an aliquot was taken for counting. When proteinuria was present, all urine samples were counted before and after precipitation of the protein with trichloroacetic acid. A correction for the excretion of unmetabolized labeled protein was made in subsequent calculations (see Appendix). All subjects received Lugol's solution, 30 drops per day orally, in order to minimize thyroidal uptake of the radioiodide. Counting was performed on 1- or 2-ml samples in a β-sensitive, plastic well scintillation counter (12).

Data and calculations

The calculations employed in this study are conventional (13) and will be briefly illustrated with reference to the data obtained from one control subject, F.W. (Figure 1).

A. The half-time of disappearance of isotopically labeled γ-globulin from serum. The half-time of disappearance is represented by the slope of the final, most nearly linear, phase of the curve obtained when serum activity per unit volume is plotted logarithmically on the ordinate against time on the abscissa. In order to compare the curves for different subjects, the daily serum activity for each subject was plotted as a fraction of the initial (10-minute) value (Figure 2).

B. Catabolic half-time. This value is obtained by plotting total nonprotein-bound urine radioactivity per 24-hour period, as for serum activity. The slope of this curve represents the rate of breakdown of the injected protein, since the iodine label is promptly excreted when catabolism takes place (13).

C. Activity retained. If urine activity is subtracted daily from the total radioactivity administered, a curve representing the activity still present in the body for each day is obtained. The slope of this curve also yields the catabolic half-time. As may be seen in Figure 1, it parallels the slope of the curve obtained directly from the urine excretion.

D. Distribution space. If the amount of activity retained in the body on any day is divided by the serum activity per unit volume for that day, the total space of distribution of the labeled protein may be calculated. This calculation is subject to several theoretical and practical difficulties, as will be shown.

RESULTS

Figure 2 presents the serum γ-globulin disappearance curves for each of the subjects in this study. All the curves showed an initial rapid decline before becoming linear at about Day 14. After this time it may be seen that all but three are parallel. These clearly show a more rapid serum disappearance (N.S.2, L.S., M.F.).

Table III presents the data for the entire group in numerical form. The patients are grouped according to the different lots of iodinated γ-globu-

TABLE II

Protein concentration and specific activity of lots of iodinated protein; dose administered to each subject

Lot no.	Date	Subject	Protein conc.	Specific activity	Amount inj.	Activity inj.
			mg/ml	*μc/mg*	*ml*	*μc*
1	7/57	G.P.	8	1.5	4.7	56.4
		N.S.			5.0	60.0
2	1/58	B.S.	20	1.7	0.78	26.7
		J.P.				
3	5/58	L.S.	20	3.8	0.74	57.0
		F.T.				
4	8/58	N.S.	20	1.2	0.99	23.8
		M.F.			1.0	24.1
		J.G.			0.56	13.5
		F.W.			1.0	24.1

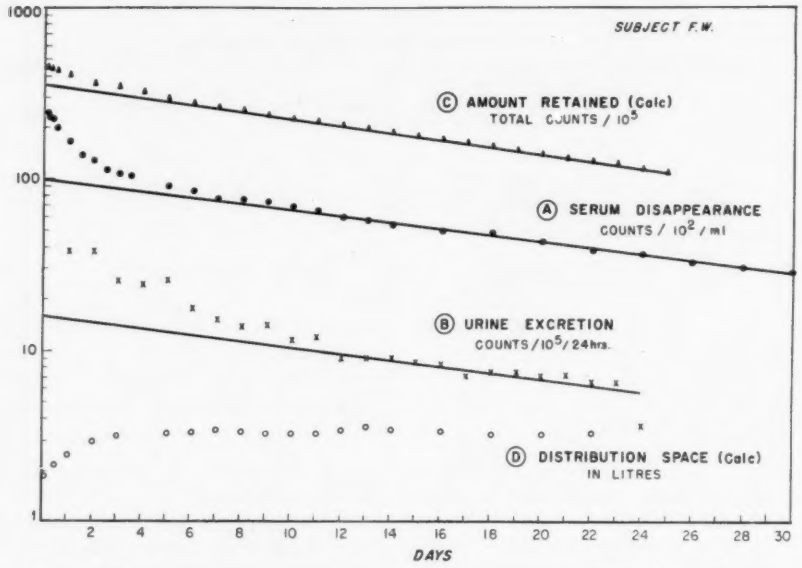

FIG. 1. PARAMETERS STUDIED IN 1 NORMAL CONTROL SUBJECT (F.W.). **A**. Daily serum activity measures as counts (in hundreds) per milliliter. **B**. Activity excreted in the urine for each 24 hour period (counts/10^5). **C**. Total activity retained in the body calculated for each day of the study (counts/10^5). **D**. Calculated space of distribution of the labeled protein (in liters).

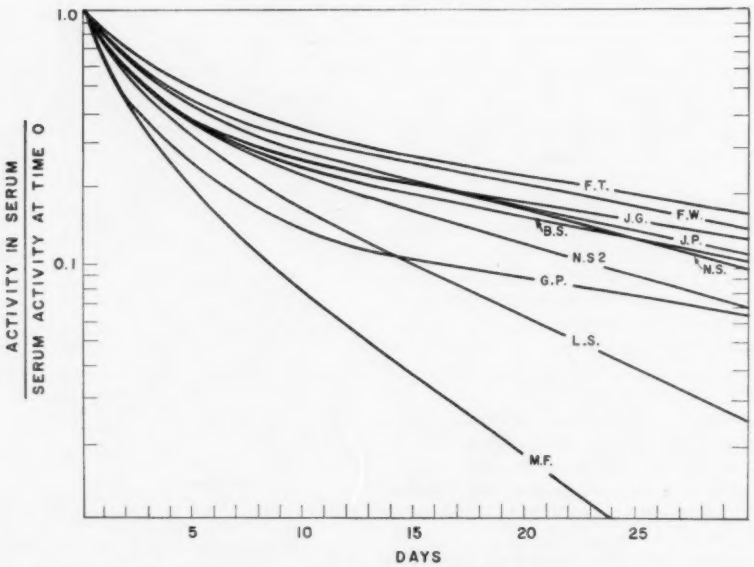

FIG. 2. CURVES OF DISAPPEARANCE OF I^{131}-LABELED γ-GLOBULIN FOR EACH SUBJECT. Plots indicate daily serum activity divided by the initial serum activity for purposes of comparison. M.F., G.P. and N.S.2 have amyloidosis. L.S., N.S., B.S. and J.P. have rheumatoid arthritis. J.G., F.W. and F.T. are normal controls.

lin used. The first group is composed of a patient (G.P.) with extensive primary amyloidosis but without renal involvement, and a patient (N.S.) with severe rheumatoid arthritis. The second group includes two subjects (B.S. and J.P.) with moderately severe rheumatoid arthritis; the third consists of a patient with severe active arthritis (L.S.) and a normal control (F.T.). The fourth group is composed of two control subjects (J. G. and F.W.), a patient with primary amyloidosis and nephrosis (M.F.), and finally, one of two patients with arthritis who had been studied in the first group and who had subsequently developed manifestations of amyloidosis with a nephrotic syndrome (N.S.).

The range of values for the half-time of globulin disappearance from the serum for the control subjects was 16.5 to 30 days. Two of the subjects with amyloidosis had values below this range and one of these, Patient N.S., studied before and after he developed clinical amyloidosis, showed a shortened half-time on both occasions. One patient with rheumatoid arthritis revealed a markedly shortened half-time of globulin disappearance. One subject with primary amyloidosis and three others with rheumatoid arthritis had disappearance times that were within the limits of the values for the control subjects.

In contrast to the values for serum disappearance, the values for the catabolic half-life of the injected protein show a wide variation. With three exceptions, however, the figures for serum disappearance and catabolic half-life are comparable. Possible explanations for the anomalous figures are discussed below. The values for distribution space as recorded in Table III do not show any significant tendencies.

TABLE III

Values for each subject of the parameters studied

Lot no.	Subject	Diagnosis	Serum half-time	Catabolic half-life	Distribution "space"
			days	*days*	*L*
1	G.P.	Amyloidosis	21	†	
	N.S.1*	Rheum. arthritis	14	†	
2	B.S.	Rheum. arthritis	19.5	12.0	9
	J.P.	Rheum. arthritis	17.5	14.5	4.5
3	L.S.	Rheum. arthritis	8.5	6.5	9
	F.T.	Control	30.	†	
4	N.S.2‡	Amyloidosis	11	9.5	8
	M.F.	Amyloidosis	4.5	3.5	6
	J.G.	Control	20	20	7.5
	F.W.	Control	16.5	16	3.5

* Patient with rheumatoid arthritis before clinical manifestations of amyloidosis.
† Determination unsatisfactory.
‡ Same patient after developing clinical amyloidosis.

DISCUSSION

The results of this study reveal three points of interest. First, they illustrate some of the complexities inherent in this method of study; second, they provide some interesting data with regard to amyloidosis; and third, they suggest a way in which rheumatoid factor might have biological activity.

The simplest parameter investigated was the rate of disappearance of the injected γ-globulin from the circulation. Although this figure is relatively free from inaccuracies of measurement, it reflects not only the rate of catabolism of the protein but also its distribution into different body compartments. For this reason the descriptive term "disappearance" has been used. It is evi-

TABLE IV

Rate of disappearance of I^{131}-labeled γ-globulin from the serum of normal humans as reported in other studies

Author	Subjects	Half-time serum disappearance	Remarks
		days	
Myant 1952 (14)	12 normal	6.0	10-day observation
Dixon and co-workers 1952 (15)	14 normal	13.1 ± 2.8	21-day observation
Eisenmenger and Slater 1953 (16)	8 normal	11.5 – 19	duration of study not stated
Havens and co-workers 1953 (17)	9 normal	10.6 – 16.6	40–60% of I^{131} excreted in first 4 days
Havens and co-workers 1954 (18)	19 normal	8–17	21-day observation
Wiener and Gordon 1957 (19)	1 agammaglobulinemia	35	unlabeled γ-globulin

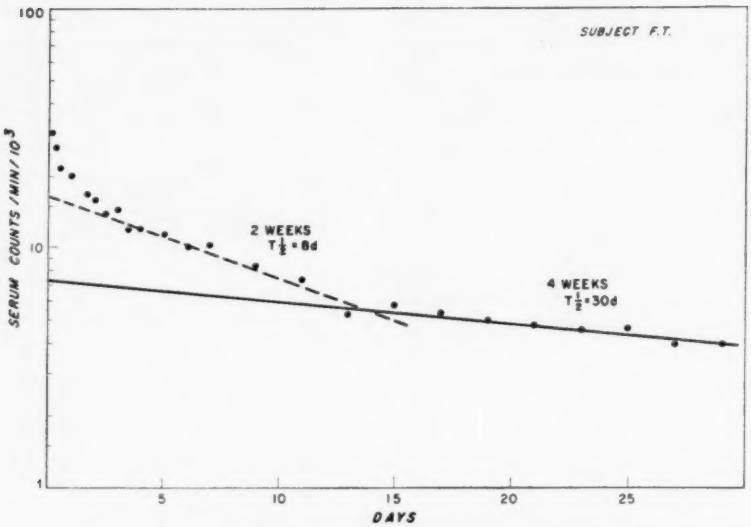

FIG. 3. EFFECT OF TERMINATING AN EXPERIMENT AT DAY 15. The solid line indicates the rate of disappearance as employed in this study. The dotted line indicates the apparent rate of disappearance had the study been terminated on Day 15.

dent that a wide range of values for this determination was encountered both in the normal subjects and in the patients studied.

A review of the literature reveals a similar spread of values for the half-time of serum globulin disappearance among normal subjects studied by different investigators (Table IV). It will be seen that the values for the control subjects in the present study are considerably longer than those recorded in Table IV. In some of these studies in which relatively short half-times of serum disappearance were found, the subjects were not followed for a period long enough to ensure that the curve plotted from the data approached a constant slope. Figure 3 illustrates the effect of a long as opposed to a short period of observation on the calculated rates of serum disappearance.

The possiblity that the labeling of proteins by radioactive iodine may alter their biological properties has been considered by several investigators (20, 21). In the preparation of the labeled protein used in this study, the ratio of moles of iodine to protein was calculated so that a maximal labeling of 3 atoms of iodine per molecule of γ-globulin was anticipated. McFarlane (22) has found that proteins lightly labeled with I^{131} have rates of catabolism similar to those of C^{14}-labeled material. Nevertheless, inferences about the metabolism of endogenous proteins from data obtained by the use of labeled protein must be guarded. The interpretation of tracer studies of this type is subject to a number of hazards. In addition to problems of sampling and measurement are those which involve the kinetics of distribution of the tracer material. These may be exceedingly complex. They have been discussed by Wrenshall (21) and by McFarlane (23). Despite these difficulties, some information of interest has been obtained.

The half-time of serum disappearance was accelerated in two of three patients with amyloidosis. One of these patients had "primary" disease; the other, pre-existing rheumatoid arthritis. Both, however, had clinical and laboratory evidence of the nephrotic syndrome. Since Gitlin and associates have shown that subjects with nephrosis from causes other than amyloidosis may have a shortened serum γ-globulin disappearance time (10), that state may have been the cause of the finding in these two patients. It is of interest that the half-time of γ-globulin disappearance was shortened in Subject N.S., both before and after he developed clinical evidence of amy-

loidosis. The pathogenesis of the shortened catabolic half-life of γ-globulin demonstrated in nephrotic subjects by Gitlin and co-workers is not known. The first study on Subject N.S. was performed 9 months before clinical evidence of nephrosis presented, and it is possible that a state of abnormal globulin metabolism was already present at that time. The fact that the third individual (G.P.), with very extensive primary amyloidosis but without nephrosis, showed a normal serum disappearance time suggests that the abnormal finding in the others was not due to amyloidosis per se.

Three of the four patients with rheumatoid arthritis showed a serum γ-globulin survival time within the normal range. It was definitely shortened in the fourth subject, L.S. Although this patient was, in general, comparable to the others, it is interesting that she is the only one with a high serum titer of rheumatoid factor. The rate of catabolism of the injected γ-globulin was similarly accelerated in this patient, showing that the rapid rate of disappearance from the serum was not due to sequestration of the protein in extravascular pools. Rheumatoid factor is capable of binding aggregated γ-globulin *in vitro* (24) and might conceivably do so when pooled γ-globulin is injected, thereby forming a complex which is more rapidly removed from the circulation and catabolized. Although, in accordance with the present evidence, this would be expected only if the injected γ-globulin were aggregated in some manner, the possible effects of the iodination procedure in this regard cannot be overlooked.[1]

The results obtained in this study may be compared with those of Vaughan and colleagues (25) who studied a group of patients with rheumatoid arthritis by a technique similar to that used here. Their results revealed a slightly but significantly more rapid rate of γ-globulin disappearance in the arthritics as compared with normal subjects. The duration of the individual studies was only 11 days, however, and for the reasons given above, this is probably too short to permit valid comparison. It is not known whether any of Vaughan's cases exhibited rheumatoid factor in high titer.

[1] Ultracentrifugal analysis showed that 94 per cent of the protein before iodination had a sedimentation coefficient of 7S.

The rate of γ-globulin catabolism agreed reasonably well with the half-time of serum disappearance in most instances in the present study. In several, however, it was very much accelerated. Technical difficulties were probably responsible for this discrepancy in three subjects—G.P., N.S.1 and F.T. Determination of the true rate of protein catabolism depends on the rate of excretion of the free radioactive marker in the urine and feces. Urine collections must be complete and continued until a constant rate of excretion is attained. In the three cases referred to above, the urine collections were unsatisfactory and the radioactivity became too low to measure accurately before either urine or serum activity had reached a constant rate of decline.

In a fourth study, B.S., in which urine collections were satisfactory, the rate of catabolism exceeded the rate of disappearance from the serum by a considerable factor. This finding can only be explained by the assumption that catabolism at a more rapid rate is taking place from some pool which is not in immediate exchange with the vascular one. While this would tend to delay the time required for the urine excretion curve to approach an asymptotic form, the difference in rates and the scatter of individual values tended to obscure this feature of the curve. It is therefore possible that the observed rate of catabolism is not the final rate which might have been attained had the experiment been prolonged.

Attempts to analyze the data from systems in which different rates of exchange occur between complexly related pools depend on certain assumptions which are difficult to prove; a fact that has limited the potential of this kind of study.

SUMMARY AND CONCLUSIONS

1. The serum survival time and catabolic half-life of intravenously injected I^{131}-labeled pooled human γ-globulin were studied in three patients with amyloidosis, four patients with rheumatoid arthritis and three normal controls.

2. The half-time of γ-globulin survival in the control subjects ranged from 16.5 to 30 days.

3. Two patients with amyloidosis, one primary and one secondary, both with the nephrotic syndrome, exhibited shortened serum half-times of 4.5 and 11 days, respectively. The serum half-

time of the latter patient, before the appearance of clinical amyloidosis, was 14 days.

4. One patient with primary amyloidosis but without nephrosis exhibited a half-time of serum γ-globulin disappearance of 21 days.

5. The half-time of γ-globulin disappearance in four patients with chronic active rheumatoid arthritis varied between 19.5 and 8.5 days. The lower figure was found in a patient having a high titer of rheumatoid factor. If this subject is excepted, the average half-time in three rheumatoid subjects is 17 days.

6. The catabolic half-life of the iodinated γ-globulin agreed in most instances with the serum half-time.

7. The calculated distribution space of the injected γ-globulin showed no consistent alteration in either amyloidosis or rheumatoid arthritis as compared with the control subjects.

8. Since the nephrotic syndrome from other causes may produce an accelerated catabolic half-life, a similar finding on our subjects cannot be ascribed to amyloidosis.

CASE HISTORIES

1. G.P. (M.G.H. no. 34 56 65). Two years prior to the study, this 55 year old bus driver noted the appearance of hemorrhagic blisters and red blotches on his eyelids, in his groins, and behind his ears. During the ensuing period he developed hypertrophic overgrowth of tissue in his auditory canals and papular masses on his tongue.

Physical examination was remarkable only in regard to the skin and tongue changes noted above. The liver and spleen were not palpable. Routine blood studies and urinalyses were negative. Biopsy of the papules on the tongue and ear and of the gingiva showed an infiltration of the skin with metachromatically staining, Congo red-positive material.

The patient died 8 months after study, probably as a result of cardiac arrhythmia. Autopsy disclosed extensive amyloid infiltration of the skin, tongue, thyroid and heart. There was no involvement of liver, spleen or kidneys.

2. N.S. (M.G.H. no. 20 57 55). This 37 year old man had experienced severe crippling rheumatoid arthritis since he was 14 years of age, and from the age of 26 was confined to bed and wheelchair.

At the time of the first study in July 1957, physical examination revealed severe deformities of hands and feet and greatly limited motion of temperomandibular, hip, knee and ankle joints, and spine. The liver and spleen were not palpable.

During the ensuing year he had a mild exacerbation of his arthritis, developed 2+ albuminuria for the first time, his liver became enlarged, and a Congo red test showed only 12 per cent retention in the serum. Gingival and renal biopsies showed changes consistent with a diagnosis of amyloidosis.

The patient developed slowly progressive uremia and died in June 1959. An autopsy revealed amyloidosis involving primarily the liver, spleen and kidneys.

3. B.S. (M.G.H. no. 52 60 05). This 60 year old woman had rheumatoid arthritis of 34 years' duration. Its course was characterized by many exacerbations and remissions initially involving the peripheral joints and, at a later date, the spine. The arthritis was accompanied by psoriasis and 3 years before study mild diabetes was discovered. She never developed rheumatoid nodules.

Physical examination revealed moderate limitation of most peripheral joints, hips and spine. The liver and spleen were not palpable. Routine blood and urine studies were not remarkable. A gingival biopsy was negative for amyloid. Congo red tests have been negative on several occasions.

4. J.P. (M.G.H. no. 78 72 05). This 61 year old woman had rheumatoid arthritis of 9 years' duration. The hands, wrists, elbows, knees, feet, temperomandibular joints and cervical spine became involved early in her course. Although the disease gradually became less active, a complete remission was never achieved. Typical rheumatoid subcutaneous nodules were present.

Physical examination disclosed moderately advanced stigmata of rheumatoid arthritis. Laboratory studies revealed normal blood indices. Urinalysis was negative except for a trace of albumin. A Congo red test showed 67 per cent retention in the serum.

5. L.S. (M.G.H. no. 74 53 16). The patient was a 62 year old housewife with rheumatoid arthritis of 8 years' duration. Starting in her hands and feet, the articular involvement spread to the knees, shoulders, cervical spine and temperomandibular joints. During two severe exacerbations she received corticosteroid therapy. Her disease was only moderately active at the time of the study and the corticosteroid therapy had been stopped 4 months previously.

Examination revealed moderately reduced mobility of the above-mentioned joints with slight periarticular swelling. Subcutaneous nodules were not present. Blood studies revealed a moderate normochromic anemia with a hemoglobin of 11.4 g. Urinalysis showed 2+ albumin and 20 to 30 white blood cells per high-power field. A Congo red test was negative, 62 per cent of the dye remaining in the serum. The latex fixation test was positive in a dilution of serum greater than 1:2,560.

6. F.T. (M.G.H. no. 67 96 90). The patient was a 63 year old laborer who had been admitted to the hospital 1 month previously because of an active duodenal ulcer and iron-deficiency anemia. Past history was negative except for pneumonia 10 years previously and chronic alcoholism.

Physical examination was unremarkable. Laboratory studies revealed no evidence of renal or hepatic dysfunc-

tion. Total serum protein was 6.6 g; albumin, 4.5 g; and globulin, 2.1 g per cent.

7. M.F. (M.G.H. no. 98 66 63). This 50 year old truck driver noted the insidious onset of edema of the feet, ankles, face and hands 1 year before the time of the study. There had been no preceding respiratory or other infections. A urinalysis 6 months prior to the onset of symptoms was normal.

The patient was admitted to the hospital 6 months before the present study. The physical examination at that time was negative except for moderate edema of the feet and hands. Routine blood studies were normal. The urinary sediment contained moderate numbers of white cells and the 24-hour protein excretion varied from 10 to 60 g. Urine culture was sterile. The renal creatinine clearance was 137 L per 24 hours.

A Congo red test revealed no retention of dye in the serum and a renal biopsy showed massive amyloidosis. A trial of prednisone therapy on two occasions produced no improvement and the drug was discontinued 3 months before the time of study.

At the commencement of this study the physical findings were essentially as described above except that the liver and spleen were just palpable. Urinary protein excretion was 8.5 and 9.8 g per 24 hours on two occasions. Sulfobromophthalein retention was 22 per cent at 45 minutes. No medications were given during the study.

8. J.G. (M.G.H. no. 27 41 01). This 48 year old man had contracted poliomyelitis at the age of 6 months. As a result he had a flail right shoulder, weak right elbow and almost flail feet bilaterally. Due to falls over the 20 years prior to admission, he sustained fractures of the right femur, left hip and left tibia, the last occurring 6 months before study. His past history was otherwise negative. At the time of study, he was up in a wheelchair undergoing a physical therapy program. Physical examination, except as above, and routine laboratory studies were entirely negative.

9. F.W. (M.G.H. no. 84 89 06). This 41 year old itinerant worker was admitted to the hospital specifically for study as a control subject. He had been well all his life except for moderate alcoholism since the age of 15.

On physical examination he appeared normal. There were no stigmata of cirrhosis. Routine blood and urine studies were negative. Liver function studies were normal.

APPENDIX

In two cases (N.S. 2 and M.F.) the excretion of protein-bound radioactivity in the urine was sufficiently great to permit accurate counting, and the half-times for serum activity disappearance were corrected by the following method.

Assumptions involved in the calculations were: *a*) intercompartmental mixing of the labeled protein is complete and takes place at rates greatly in excess of the combined rates of excretion of protein and nonprotein activity into the urine, and *b*) the concentration of free radioiodide in the blood is negligible. Therefore,

$$Ct = Co\, e^{-[k_1+k_2]t}$$

where Ct = the observed serum activity at any time t after mixing is complete; Co = the serum activity at $t = 0$; k_1 = the rate constant for the loss of protein-bound activity into the urine; k_2 = the rate constant for the loss of free radioiodide into the urine.

The values for each of these constants may be determined by the relation

$$k_1/k_1 + k_2$$

= urine protein-bound activity/total urine activity

for any given period after mixing is complete.

From these data k_2 may be determined and a corrected half-time of disappearance of iodinated γ-globulin from the serum computed.

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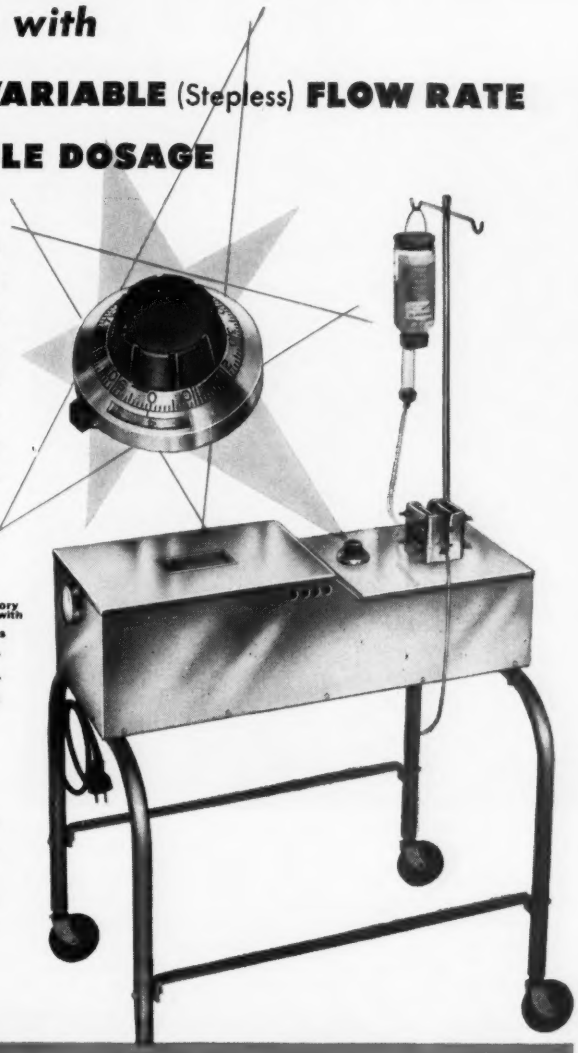

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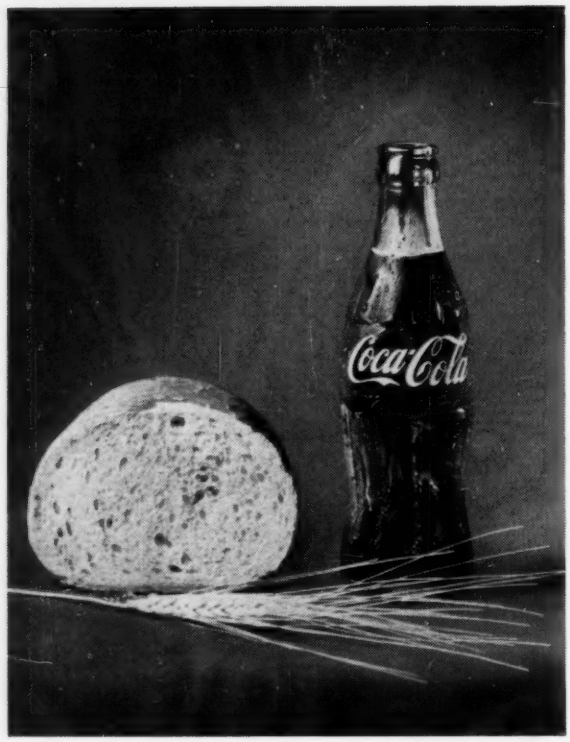

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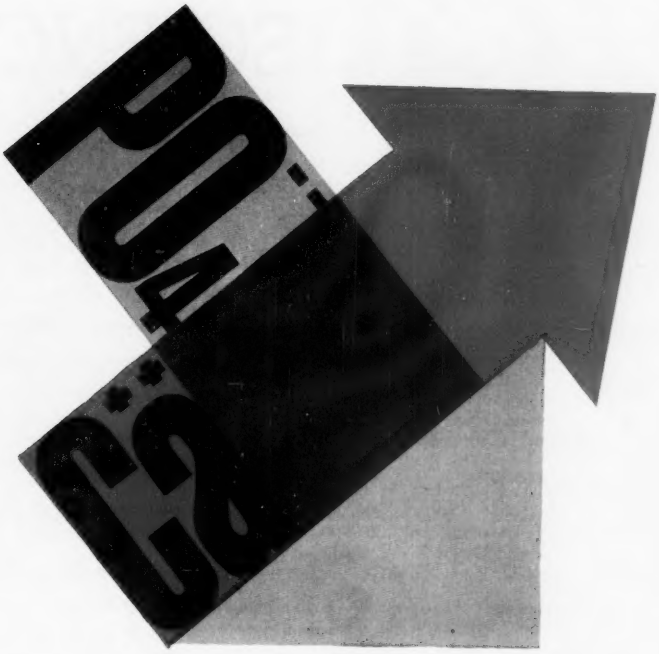

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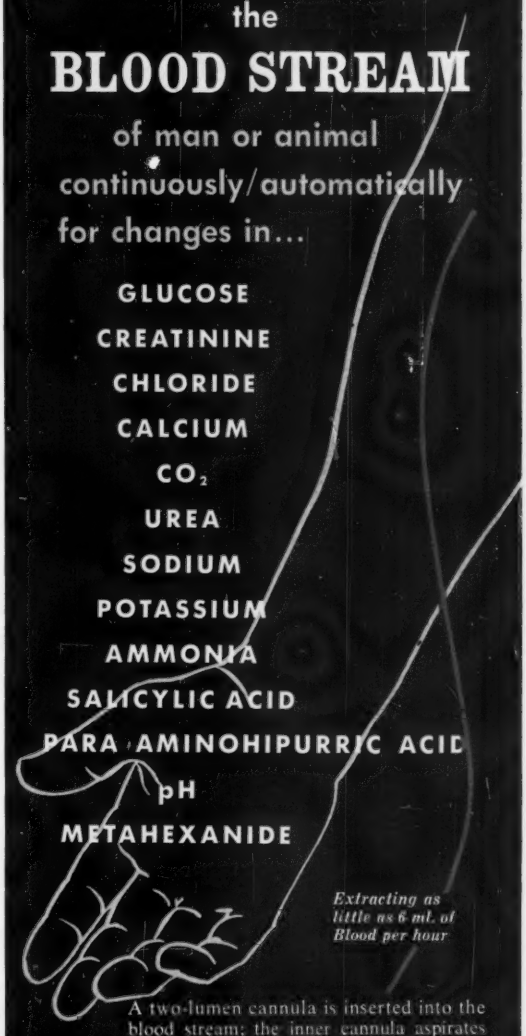

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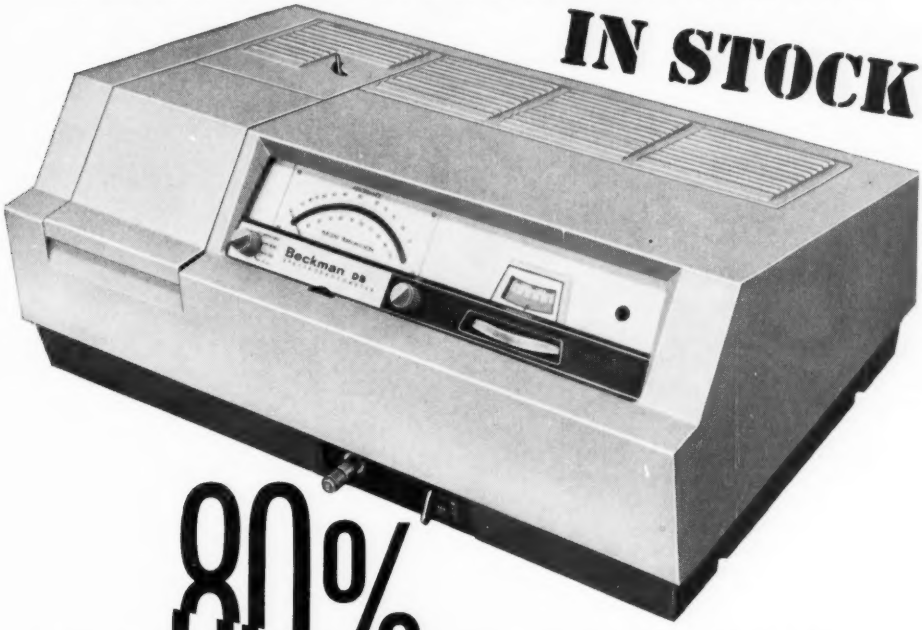

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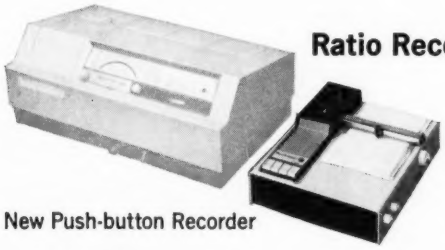

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